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FOURTH SYMPOSIUM ON SUBTYPES OF MUSCARINIC RECEPTORS

FINAL REPORT

RUTH R. LEVINE

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SCIENTIFIC REPORT: FOURTH INTERNATIONAL SYMPOSIUM ON SUBTYPES OF MUSCARINIC RECEPTORS

Twenty-one papers of 20-minute duration were presented—> during the three day period of the Symposium. All of these papers and the abstracts of 76 posters have been published in the Proceedings (attached) which appeared as a supplement to the December, 1989 issue of Trends in Pharmacological Sciences (TIPS). Over 200 names are associated with the authorship of the papers and with that of the poster abstracts.

A total of 186 invited scientists registered and attended the Symposium (names and addresses of pre-registrants are attached). The attendance at each of the sessions was excellent and appeared to include all registrants. The 10 minute discussion period between papers was lively, provocative and informative. The posters were available for viewing for the entire meeting and were manned each afternoon because there was so much enthusiasm for the work being presented.

The Proceedings of the Symposium was sent to about 5,000 scientists, a number which included all subscribers to TIPS and all active participants in the symposium.

Grant support from the U. S. Army Medical Research and the Development Command has been acknowledged in the published Proceedings.

The comments of participants at the Symposium and those received in response to the Proceedings indicate great interest in the program in general and in the specific topics and papers of the Symposium. It appears quite evident that the Fourth Symposium on Subtypes of Muscarinic Receptors has stimulated and is stimulating additional research which should, indeed, lead to the development of new and better therapeutic agents as well as agents useful to the U. S. Army Research and Development Command.

As another mark of the success of the Fourth Symposium, the attendees request a Fifth Symposium to be held in two to three years. Planning for another symposium was, therefore, initiated at the meeting.
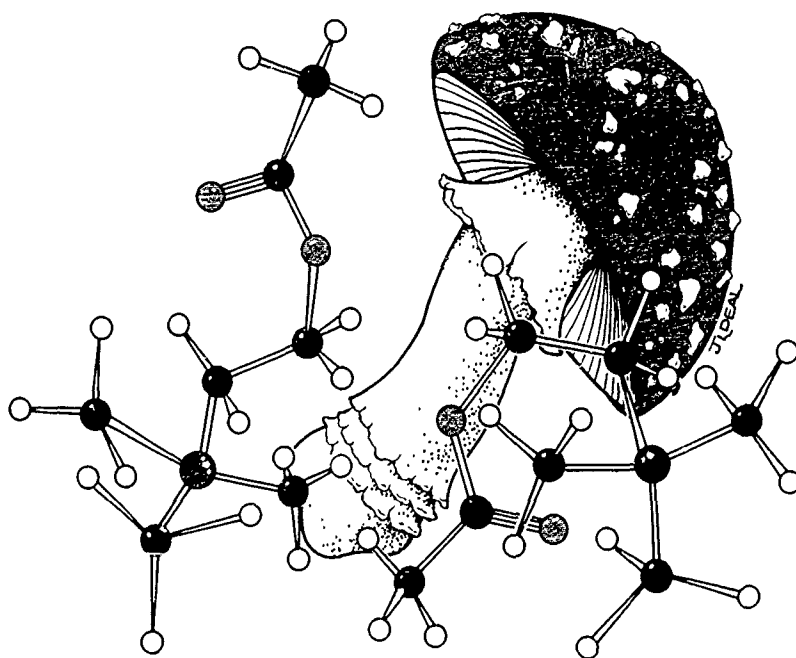
Subtypes of Muscarinic Receptors IV

Proceedings of the
Fourth International Symposium
on Subtypes of Muscarinic Receptors

Editors
Ruth R. Levine and Nigel J. M. Birdsall

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**20–22 July 1989
Wiesbaden, FRG**

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Contents

Topes includes.)

Acknowledgements

Nomenclature for muscarinic receptor subtypes recommended by symposium

Three subtypes of muscarinic receptor, M_1, M_2 and M_3, may be defined on the basis of the action of antagonists. It should be noted that none of the antagonists in the table below is highly selective for one receptor subtype over all other subtypes. Hence the pharmacological characterization of a muscarinic receptor subtype should be based on its spectrum of affinities for a number of antagonists rather than on the basis of its affinity for a single antagonist. Attention should be given when characterizing receptor subtypes to the possibility that more than one receptor subtype may be present in the tissue examined.

At the present time the sequences of five muscarinic receptor species (m1–m5) are known. Some species vary slightly in their length, depending on the mammalian species from which they were cloned. The sequence of a specific mammalian species may be denoted by an upper case prefix, e.g. Hm1 for the human m1 sequence. Other prefixes in current use are: R, rat; P, pig; M, mouse; C, chicken; D, *Drosophila*.

It is likely, but not unambiguously proven, that the m1 sequence corresponds to that of the M_1 receptor, m2 to the M_2 receptor and m3 to the M_3 receptor. There is a candidate M_4 receptor, found in the striatum and some cell lines (e.g. NG108-15), which inhibits adenylate cyclase. The pharmacology of this receptor, as well as that of the expressed m4 and m5 species, whilst being different from M_1, M_2 and M_3 receptors, is not sufficiently distinctive or well characterized to allow an unambiguous assignment of a pharmacological M_4 (or M_5) receptor subtype.

Nomenclature committee: Nigel Birdsall (chairman), Noel Buckley, Henri Doods, Kazuhiko Fukuda, Antonio Giachetti, Rudolf Hammer, Heinz Kilbinger, Guntei Lambrecht, Ernst Mutschler, Neil Nathanson, Alan North and Roy Schwarz.

Muscarinic receptor nomenclature

Pharmacological characterization					
Subtype	M_1	M_2	M_3	–	–
Other names used previously	$M_{1\alpha}$, A	$M_{2\alpha}$, cardiac M_2, C	M_2, $M_{2\beta}$, B, glandular M_2	M_2	–
Selective antagonists	pirenzepine, (+)-telenzepine	AF-DX 116, himbacine, methoctramine, gallamine*	*p*-fluorohexa-hydrosila-difenidol, hexahydrosila-difenidol	–	–
	$M_1>M_3\geqslant M_2$	$M_2>M_1\geqslant M_3$	$M_3\geqslant M_1>M_2$		
Molecular characterization					
Sequences	m1	m2	m3	m4	m5
Other names used previously	mAChRI, M1	mAChRII, M2	mAChRIII, M4	mAChRIV, M3	
Numbers of amino acids	460	466	589/590	478/479	531/532

* not competitive

Some considerations of receptor specificity

Sir Arnold S. V. Burgen

Since the amino acid sequences for several muscarinic and adrenergic receptors are known, an attempt was made to correlate side chain hydrophobicity surrounding the aspartate anionic groups with binding structure–activity relationships. No positive correlation was found, suggesting that secondary binding effects of head group substituents are non-local. Displacement of aspartate ionization is unlikely to be due to such neighbour effects.

The growth of structural information on drug receptors begs the question whether it yet provides any insights into problems of structure–activity relationships and receptor specificity. The sequence data available on several subtypes of the muscarinic, adrenergic, dopamine and serotonin receptors show striking sequence identities and homologies that suggest they belong to a single family of proteins derived from a common prototype.

Based on the distribution of hydropathy they are all considered to have a similar structure to that determined for bacteriorhodopsin at low resolution by electron microscopy. That is that they comprise seven transmembrane α-helical segments coupled by segments on both extracellular and cytoplasmic faces, about which useful conformational predictions cannot be made at present. Within the presumed helical regions the sequence homologies are especially strong. These transmitters are cations and for all these receptors there is evidence that an important part of the energy of interaction of the ligand with the receptor is through ion-pairing with an anionic residue, i.e. an aspartate or glutamate. Evidence has been adduced for both muscarinic receptors and adrenoceptors that the residues concerned are aspartates in the second and/or third helices[1-3].

Remarkably, two aspartates near the middle of these helices have been conserved throughout the series of receptors mentioned[4]. While one must be very cautious in assuming that the seven-helix model is anything other than a good model of the receptor structure, it nevertheless encourages one to ask whether there is anything about the environment of these two aspartates that can explain some of the known structure-activity relationships at muscarinic receptors and adrenoceptors.

Among muscarinic agonists it is established for all agonists in which the basic nitrogen is not part of a cyclic structure, that the quaternary trimethylammonium head provides the optimum structure. For cyclic structures the tertiary amine may be the most active. This is illustrated with the acetylcholine series[5] in Table I. Removal of methyl groups progressively reduces activity and replacement by ethyl groups is also disfavoured. This suggests that the optimal interaction

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is not of the salt bridge structure which is found in the interior of some proteins, i.e.:

$$— COO^- \cdots\cdots\ ^+NH_3 —$$

but that the ionic bond is reinforced by non-polar interactions between the N-methyl groups and non-polar residues in the receptor, thus:
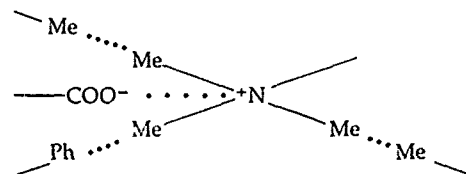
These interactions are presumably sterically interfered with by the larger alkyl groups.

A different situation is found in the adrenoceptors[6] (Table II). Here the quaternary derivative is very weak and the optimum structure is monomethylated. This is only slightly favoured over the primary amine in the α-receptor, but strongly favoured in the β-receptor which also accepts larger alkyl groups without much loss of activity. Histamine H_1 receptors are not dissimilar although the primary amine is by a small margin the most active.

Since these groups attached to the cationic head will be in the neighbourhood of the aspartate during interaction with the receptors, it is not unreasonable to suggest that the side chains of amino acids which in the α-helix form the neighbours of the aspartate, will be groups immediately available for interaction with the N-alkyl groups.

The hypothesis then suggests that the interaction of acetylcholine with the muscarinic receptor might be

TABLE I Relative activity of acetylcholine homologues

$CH_3COOCH_2CH_2\overset{+}{N}Me_3$	1		
—$\overset{+}{N}Me_2H$	0.02	—$\overset{+}{N}Me_2Et$	0.3
—$\overset{+}{N}MeH_2$	0.002	—$\overset{+}{N}MeEt_2$	0.002
—$\overset{+}{N}H_3$	0.0001	—$\overset{+}{N}Et_3$	0.0005

TABLE II. Relative activity of homologues of noradrenaline at α- and β-adrenoceptors

	α	β
$(3,4\text{-OH})\text{-PhCHOH-CH}_2\overset{+}{N}Me_3$	<0 001	<0 001
————$\overset{+}{N}HMe_2$	0.025	J 001
————$\overset{+}{N}H_2Me$	1	1
————$\overset{+}{N}H_3$	0 5	0.005
————$\overset{+}{N}H_2Et$	0 08	1
————$\overset{+}{N}H_2\text{-}iso\text{-Pr}$	0.0001	0.16

favoured by hydrophobic amino acid residues, whereas in the adrenoceptor these residues will not be required. Since an α-helix undergoes a single turn for every 3.6 residues, the neighbours will be residues 3 and 4 places above and below the aspartate. Figure 1 shows the clusters around these aspartates[4] in helix II and helix III.

Helix

Fig. 1. Aspartate residues in helices II and III with the surrounding cluster of amino acid side chains.

In helix II of the muscarinic receptors three of the residues are strongly hydrophobic whilst the remaining residue is a glycine which, lacking a side chain, does not contribute. The residues are identical in all five receptor subtypes. However, the cluster in the β-receptor has a very similar pattern and hydrophobicity. In the α_2-receptor all four residues are hydrophobic, but in α_1-receptor one of the residues is a hydrophilic serine. There is nothing here that correlates with the ligand preferences.

The situation with helix III is rather different, in that two of the residues in the muscarinic receptor are Trp and Ser which are weakly hydrophilic and there is a difference between m1, m3 and m5 on one hand and m2 and m4 on the other; a similar balance is found in the β-receptors, while the α-receptors have only a single hydrophilic residue. The immediate environment of the aspartates in helix III does not help either in discriminating between the two receptors. Our conclusion is that the important hydrophobic interactions of the N-methyls in the muscarinic receptor must not be with the hydrophobic groups on helix II and III.

There are two other aspartates in helix III that may be relevant to the binding site. One of them is at the presumed cytoplasmic end of the helix (H IIIc, residue 122 in m1). Once more the arrangement of the hydrophobic residues (Fig. 2) does not help in discriminating between simple charge interactions and possible interactions reinforced by nonpolar interactions. It is interesting that once again these residues do discriminate between m1, m3 and m5, and m2 and m4 and these two sets of conserved residues seem likely to have some role in determining the selectivity of the subtypes.

The other aspartate is very close to the extracellular end of helix III (H IIIe; residue 99 in m1). In this case the residues further into the helix are fully conserved in all five receptor subtypes (Fig. 3), but the residues extending out of the helix are not conserved. The comparison with the adrenoceptor is complicated by the replacement of the aspartate by glutamate in β_1, β_2 and α_2, and by the presence of an arginine in α_1.

The residues about the β_1, β_2 aspartate are more hydrophilic and so to a lesser extent are those about α_2. The situation in α_1 means that the aspartate will be ion paired with the arginine and would not provide a satisfactory anionic partner for the catecholamine.

It is apparent that no simple rules relating circum-aspartate residues to the problem of amine alkyl states in the agonists can be deduced.

Perhaps there should be little surprise at this conclusion because we know that even tetramethyl-ammonium is an agonist and as such must be capable of initiating a concerted conformational change in the receptor and, therefore, must be interacting with at least two subsites on different helices.

It should also be recalled that in muscarinic antagonists, the effects of alkylation are different from those in agonists. For instance in benzilic esters[7] while demethylation reduces activity, ethyl replacement has negligible effects (Table III). This implies less constraint in the site in the conformation complementary to antagonists.

It was originally found by C. R. Hiley (PhD thesis,

Fig 2 residue diagram:

```
        Leu        Ile          Ile        Ile
m1 }
m3 }   [Asp]      m2 }        [Asp]
m5 }              m4 }
        Phe        Ser          Phe        Cys


        Ala        Ile          Val        Ile
β₁     [Asp]      β₂          [Asp]
        Leu        Ala          Ala        Ile


        Ala        Ile          Ala        Ile
α₁     [Asp]      α₂          [Asp]
        Ile        Gly          Trp        Ser
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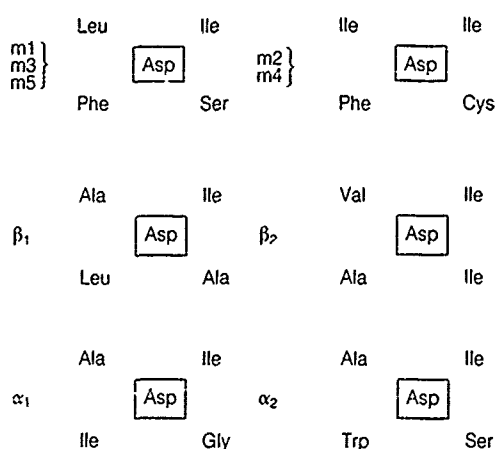
Fig 2. Residues around aspartate H IIIc.

University of Cambridge, 1974) that the anionic group in the receptor had an apparent pK of 6.2. This is somewhat higher than would be expected for the free β-carboxyl of aspartate in a peptide.

It was suggested by Wheatley et al.[1] that this was due to the environment of the carboxyl being hydrophobic. It is doubtful if the adjacent residues on the helix in any of the aspartates could provide sufficient hydrophobicity to account for this; furthermore, the apposition of

Fig 3 residue diagram:

```
       Leu   Ala            Leu   Ala
m1    [Asp]         m2     [Asp]
       Leu   Thr            Val   Pro

       Leu   Ser            Leu   Ala
m3    [Asp]         m4     [Asp]
       Leu   Asn            Val   Ala

       Leu   Ala
m5    [Asp]
       Leu   Ser

       Thr   Ser            Thr   Ser
β₁    [Glu]         β₂     [Glu]
       Phe   Ser            Phe   Asn

       Ala   Ala            Leu   Ala
α₁    [Asp]         α₂     [Glu]
       Ile   Arg            Thr   Trp
```

Fig. 3. Residues and aspartate H IIIe.

hydrophobic groups close enough to the carboxyl to complete a hydrophobic shell would not seem to allow room for the interaction of a ligand. It is also well known that free charged groups are not normally found in the interior of proteins, since they would be energetically disfavoured. However, the high dielectric constant of water, which is responsible for the strength of the carboxyl group in water, is related to the high mobility of water dipoles. In many proteins, water molecules in crevices form structural networks, and because their motion is partly frozen, the effective dielectric constant is substantially reduced below that in liquid water[8] and this could account for the weaker ionization of the aspartate. A further factor that must be taken into account is that while the helices are singularly free of basic residues except near their cytoplasmic terminators, the aspartates in helices II and III may be sufficiently close in the aqueous channel in which the ligand inserts to be mutually interactive, as they would be in a polycarboxylic acid. The effect could be to shift the ionization of the carboxyls so that some might have a lower pK and others a higher pK than in an unperturbed aspartate peptide.

TABLE III. Activity of benzilyl esters

Ph₂C(OH)COCH₂CH₂ṄMe₃	1
——————————Me₂H	0.10
——————————Me₂Et	1.8
——————————MeEt₂	2.3
——————————Et₃	1.8

Our conclusion must be that it is premature to make deductions about specificity from the structural information so far available. Further clues will undoubtedly come from the genetic engineering of mutant and hybrid receptors. But, even when full three-dimensional structures of proteins are available, it is by no means straightforward to interpret patterns of ligand interaction, so we should keep our expectations in check.

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Selective effector coupling of muscarinic acetylcholine receptor subtypes

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Attempts have been made by means of recombinant DNA technology to understand the molecular basis of the functional heterogeneity of the muscarinic acetylcholine receptor (mAChR). Molecularly defined mAChR subtypes have been produced from the cloned DNAs in Xenopus oocytes and NG108-15 neuroblastoma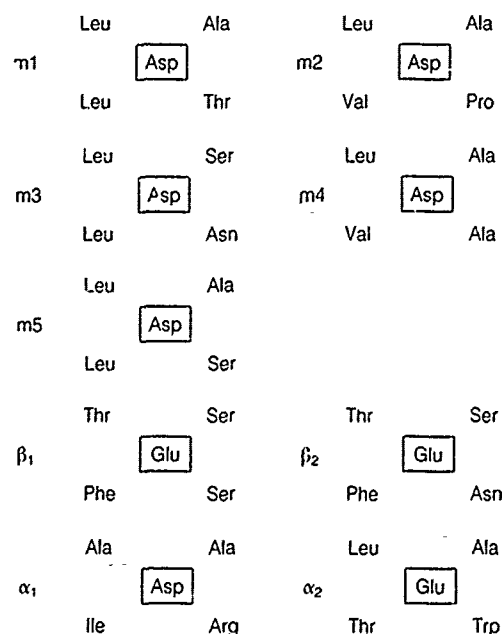–glioma hybrid cells as transient and stable expression systems, respectively, and agonist-induced cellular responses have been examined. The results obtained provide evidence that mAChR subtypes are selectively coupled with different effector systems, albeit not exclusively.

The muscarinic acetylcholine receptor (mAChR) mediates a variety of cellular responses, including inhibition of adenylate cyclase, breakdown of phosphoinositides and modulation of potassium channels, through the action of G proteins[1]. An important issue is to elucidate the molecular mechanisms underlying the various biochemical and physiological effects. Pharmacologically distinguishable forms of the mAChR occur in different tissues and have been classified into M_1, M_2 and M_3 subtypes on the basis of their difference in apparent affinity for antagonists[2-4]. An effective approach to studying the functional heterogeneity of the mAChR has been provided by recombinant DNA techniques. The primary structures of two mAChR species, designated as m1 and m2, have been elucidated by cloning and sequence analysis of DNAs complementary to the porcine cerebral and cardiac mRNAs, respectively[5-7]. The primary structures of two additional mAChR species, designated as m3 and m4, have subsequently been deduced from the nucleotide sequences of the cloned cDNAs or genomic DNAs[8-10]. A fifth mAChR species (m5) has further been identified by DNA cloning[11,12]. The hydropathy profiles of the five mAChRs are similar and suggest the presence of seven transmembrane segments (I–VII) as in the case of other G protein-coupled receptors. The present paper deals with our attempts to understand the molecular basis of the functional heterogeneity of the mAChR using DNA expression systems.

Molecular basis for pharmacologically defined mAChR subtypes

mRNAs specific for the four individual mAChR species (m1–m4) were synthesized by transcription *in*

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vitro of the respective cDNAs or genomic DNAs (containing no introns in the protein-coding region) linked with the bacteriophage SP6 promoter and were injected into *Xenopus* oocytes to yield functional mAChRs[5,10,13,14]. The ligand-binding properties of the four mAChRs expressed in oocytes were examined using cell extracts. The apparent K_d for $(-)[^3H]QNB$, estimated by Scatchard analysis, was similar for all the mAChRs. The apparent K_d values for selective antagonists were obtained by measuring displacement of $(-)[^3H]QNB$ binding by increasing concentrations of the antagonists. The results of these experiments (Table I) indicate that m1, m2 and m3 correspond most closely to the pharmacologically defined M_1^2, M_2^3 and $M_3^{4,15\ 16}$ subtypes, respectively.

The tissue distribution of the mRNAs encoding m1, m2, m3 and m4 was investigated by blot hybridization analysis with specific probes[5,6,9,17] (Fig. 1). All four mAChR mRNAs are present in cerebrum, whereas only m2 mRNA is found in heart. Exocrine glands contain both m1 and m3 mRNAs, whereas smooth muscles contain both m2 and m3 mRNAs. Thus the mAChR heterogeneity in tissues with respect to antagonist binding can be accounted for by the presence of individual molecularly distinct mAChR species or various combinations of them.

Differential cellular responses mediated by mAChR subtypes

With the aim of answering the question of whether the different molecularly defined mAChR subtypes are coupled with distinct effector systems, agonist-induced responses mediated by the individual mAChR species were investigated using *Xenopus* oocytes and NG108-15 neuroblastoma–glioma hybrid cells[18] as transient and stable expression systems, respectively[5,13,14,19,20].

Responses in Xenopus oocytes

Xenopus oocytes injected with the mRNA specific for m1, m2, m3 or m4 were examined for electrophysiological responses induced by ACh under voltage clamp at a holding potential of −70 mV. Figure 2 shows typical

TABLE I. Apparent K_d of mAChR subtypes for antagonists

| Antagonist | $K_d(M)$ | | | | |
	m1 (porcine)	m2 (porcine)	m3 (porcine)	m3 (rat)	m4 (rat)
(−)[³H]QNB	8.4×10^{-11}	1.3×10^{-10}	1.2×10^{-10}	1.0×10^{-10}	9.5×10^{-11}
Pirenzepine	1.8×10^{-8}	6.6×10^{-7}	1.8×10^{-7}	1.3×10^{-7}	1.2×10^{-7}
AF-DX 116	2.5×10^{-6}	7.3×10^{-7}	2.3×10^{-6}	3.1×10^{-6}	2.3×10^{-6}
Hexahydrosiladifenidol	5.1×10^{-8}	2.8×10^{-7}	4.4×10^{-9}	4.0×10^{-9}	2.0×10^{-8}

Reprinted from Ref 10

ACh responses observed in oocytes injected with the mRNA specific for each of the four mAChR subtypes. Oocytes implanted with m1 or m3 exhibited an oscillatory inward current (Fig. 2A, C), whereas a typical ACh response induced in oocytes implanted with m2 or m4 comprised an initial smooth inward current followed by an oscillatory component (Fig. 2B, D). Application of hyperpolarizing test pulses indicated that these inward currents were accompanied by increased membrane conductance. The oscillatory current evoked by activation of m2 or m4 varied in amplitude among the oocytes tested and was undetected in some of them. The latency of the ACh response in m2- or m4-implanted oocytes was shorter than that in m1- or m3-implanted oocytes, the former being mostly attributable to the dead-space time in the perfusion system (~ 7 s). The current response in m1- or m3-implanted oocytes occurred after an additional delay (2–3 s).

The reversal potential of the oscillatory current induced by activation of each of the four mAChR subtypes, obtained in Ringer's solution, was around −25 mV, which is close to the equilibrium potential of Cl⁻ in Xenopus oocytes[21]. This suggests that the oscillatory current is carried mainly by Cl⁻. In contrast, the smooth current mediated by m2 or m4 was reversed in polarity at a potential around 10 mV. This value does not correspond to the equilibrium potential of any single species of ions, indicating the involvement of more than one ion species in the generation of the smooth current. Reversal potential measurements in different media performed with EGTA-loaded oocytes (see below) suggest that the smooth current evoked by activation of m2 or m4 is carried principally by Na⁺ and K⁺. Intracellular injection of the Ca^{2+}-chelating agent EGTA almost completely abolished the ACh-activated current in m1- or m3-implanted oocytes, leaving only a small long-lasting inward current. The oscillatory current in m2- or m4-implanted oocytes similarly disappeared after this treatment, whereas the smooth component was virtually unaffected. Thus our results provide evidence that m1 and m3 mediate activation of a Ca^{2+}-dependent Cl⁻ current, whereas m2 and m4 principally induce activation of Na⁺ and K⁺ currents in a Ca^{2+}-independent manner. Most likely, the activation of the Cl⁻ current mediated by m1 and m3 results from phosphoinositide hydrolysis leading to Ca^{2+} release from intracellular stores.

Responses in NG108-15 cells

NG108-15 cells were transfected with cDNA or genomic DNA encoding each of the four mAChR subtypes, using an expression vector carrying the SV40 early gene promoter and the neomycin-resistance
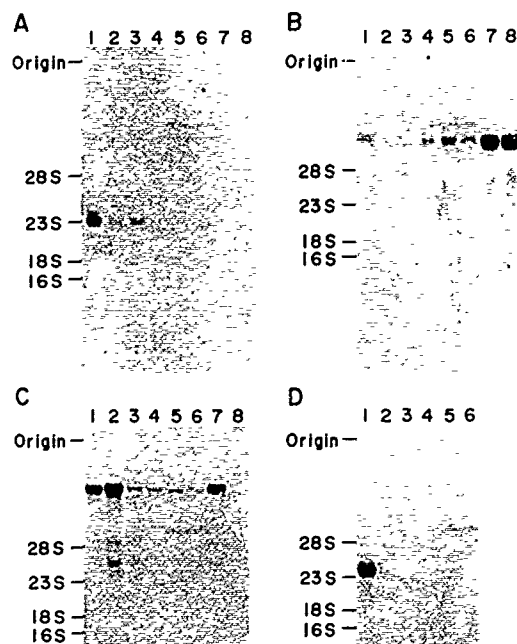
Fig. 1. Autoradiograms of blot hybridization analysis of poly(A)⁺ RNA from porcine (A–C) and rat tissues (D) using probes specific for the mRNAs encoding porcine m1 (A), porcine m2 (B), porcine m3 (C) or rat m4 (D) A–C. Analysis of poly(A)⁺ RNA (15 µg each) from porcine cerebrum (lane 1), lacrimal gland (lane 2), parotid gland (lane 3), small intestine (lane 4), large intestine (lane 5), trachea (lane 6), urinary bladder (lane 7) and atrium (lane 8) The rat RNA species hybridizable with the rat m3-specific probe showed a similar tissue distribution D Analysis of poly(A)⁺ RNA (15 µg each) from rat cerebrum (lane 1), submandibular gland (lane 2), small intestine (lane 3), trachea (lane 4), urinary bladder (lane 5) and heart (lane 6) From Ref 17.
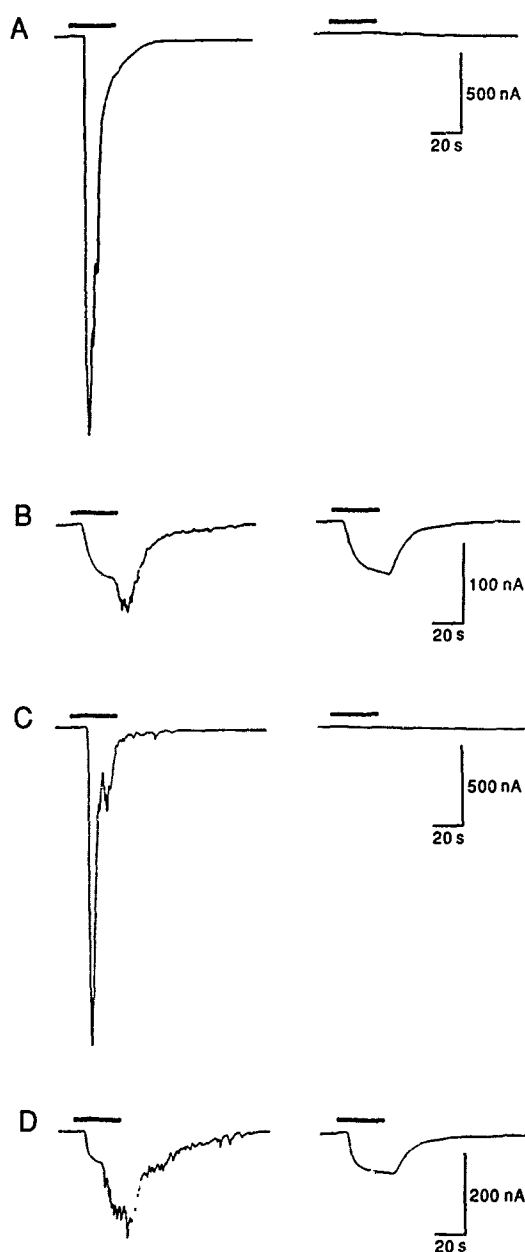
Fig 2 ACh-activated currents in Xenopus oocytes injected with the mRNA specific for porcine m1 (A), porcine m2 (B), porcine m3 (C) or rat m4 (D). Whole-cell currents activated by bath application of 1 μM (A, B, D) or 10 nM ACh (C) were recorded under voltage clamp at a holding potential of −70 mV before (left) and after (right) intracellular injection of EGTA. Inward current is downward. The duration of ACh application is indicated by bars without taking into account the dead-space time in the perfusion system (~7 s) The ACh response in oocytes injected with the rat m3-specific mRNA was similar to that in oocytes injected with the porcine m3-specific mRNA. From Ref. 13 (A, B) and Ref. 14 (C, D).

marker gene[19]. The expression of each mAChR subtype was confirmed by (−)[³H]QNB binding assay and by blot hybridization analysis of total cellular RNA using mAChR subtype-specific probes. NG108-15 cells endogenously contain the m4 mRNA[9,19].

The transformed clones were tested for electrophysiological response to ACh under voltage clamp at a holding potential of −30 mV. Cells transformed to express m1 or m3 exhibited a similar response to ACh, which comprised an initial outward current followed by a sustained inward current. The initial outward current was accompanied by an increase in input conductance, as measured by short voltage steps (Fig. 3A, B). The outward current was reversed to an inward current on hyperpolarizing cells to −73 to −85 mV and the current–voltage (I–V) relation was apparently linear (Fig. 3C). The outward current response was reduced or abolished by apamin (0.4 μM) or (+)tubocurarine (0.2 mM), but was resistant to tetraethylammonium (1 mM). These results indicate that the initial outward current is principally attributable to activation of a subclass of Ca^{2+}-dependent K^+ currents.

Unlike the initial outward current, the secondary inward current induced by ACh was usually accompanied by a decrease in input conductance and by a reduction in the time-dependent inward current relaxation observed during hyperpolarizing voltage steps (Fig. 3A, B). The steady-state I–V curve that was obtained while the sustained inward current was present showed a reduction in the outward rectification in a potential range less negative than approximately −70 mV (tested up to −20 mV), where the M current[22,23] is activated (Fig. 3C). These data indicate that the secondary inward current results primarily from inhibition of the M current, which is known to be present in NG108-15 cells, some neuronal cells and smooth muscle and to be modulated by different neurotransmitters[24].

The initial outward current was observed in 88% and 81% of the m1- and m3-transformed cells tested, respectively. The secondary inward current occurred in 59% and 64% of the m1- and m3-transformed cells examined, respectively. In contrast, the percentages of responsive cells among the m2- or m4-transformed cells (12% for the outward current and 6% for the inward current), despite their (−)[³H]QNB binding activities being comparable to those of the m1- or m3-transformed cells, were not higher than those of nontransfected or vector-transformed control cells (14% and 6%, respectively).

The effect of carbamylcholine on the formation of inositol phosphates was next examined using [³H]inositol-labelled cells. In m1- or m3-transformed cells, a four- to seven-fold increase in the release of total inositol phosphates occurred in response to carbamylcholine stimulation, as compared with control values obtained without stimulation. But in m2- or m4-transformed cells, as well as in nontransfected or vector-transformed cells, no appreciable increase in inositol phosphate release was observed. Studies with the fluorescent indicator dye fura-2 showed that m1 and m3 expressed in NG108-15 cells, unlike m2 and m4, mediate release of Ca^{2+} from intracellular stores[20].

These results indicate that the molecularly defined

m1 and m3 subtypes, but not the m2 and m4 subtypes, efficiently mediate phosphoinositide hydrolysis, release of Ca^{2+} from intracellular stores, activation of a Ca^{2+}-dependent K^+ current and inhibition of the M current in NG108-15 cells. It is suggested that the m4 subtype mediates adenylate cyclase inhibition without affecting phosphoinositide hydrolysis in NG108-15 cells[25]. Stable expression of the four mAChR subtypes in other mammalian cell lines also shows that m1 and m3 are coupled with stimulation of phosphoinositide hydrolysis[26-28], whereas m2 and m4 are linked mainly with inhibition of adenylate cyclase[26,28,29]. Furthermore, the fifth mAChR species (m5) has been shown to be coupled with phosphoinositide hydrolysis efficiently[11,12]. These findings, together with our DNA expression studies using *Xenopus* oocytes, suggest that mAChR subtypes are selectively coupled with different effector systems, albeit not exclusively. The inhibition of the M current mediated by m1 and m3 suggests that these subtypes are involved in the cholinergic excitation of vertebrate neurons evoked by muscarinic activity.

Different agonist sensitivities of mAChR subtypes

Figures 4A and 4B show the effects of increasing concentrations of agonist on the formation of total [³H]inositol phosphates and on intracellular Ca^{2+} release, respectively, in NG108-15 cells expressing m1 or m3. The EC50 value for m3-transformed cells was almost 10-fold smaller than that for m1-transformed cells. Figure 4C shows dose–response relationships for ACh-activated peak inward currents obtained from oocytes implanted with m1 or m3. To elicit a current of similar amplitude, m1 required a ~ 30-fold higher concentration of ACh than did m3. These data reveal that m3 has a sensitivity to agonist of about one order of magnitude higher than that of m1 in stimulating phosphoinositide hydrolysis and intracellular Ca^{2+} release in NG108-15 cells and in mediating activation of a Ca^{2+}-dependent Cl^- current in *Xenopus* oocytes. In the experiment shown in Fig. 4D, the agonist binding affinities of m1 and m3 were examined using oocyte extracts. The apparent K_d for carbamylcholine of m3, obtained by measuring displacement of $(-)[^3H]QNB$ binding, was about one order of magnitude lower than that of m1. This result suggests that the higher sensitivity to agonist of m3 is probably attributable, at least partly, to its higher agonist-binding affinity.

Location of a region of mAChR involved in selective effector coupling

In an attempt to localize the region of the mAChR molecules responsible for selective coupling with different effector systems, chimaeric mAChR molecules with different combinations of m1 and m2 were produced in *Xenopus* oocytes by expression of the corresponding cDNAs and were compared with respect to ACh-induced current responses and antagonist-binding properties[30]. Figure 5A shows the ACh-induced current responses at -70 mV membrane potential observed in oocytes implanted with the different chimaeric mAChRs and with the parental m1 and m2 before and after intracellular injection of EGTA. The ACh-activated inward current mediated by the
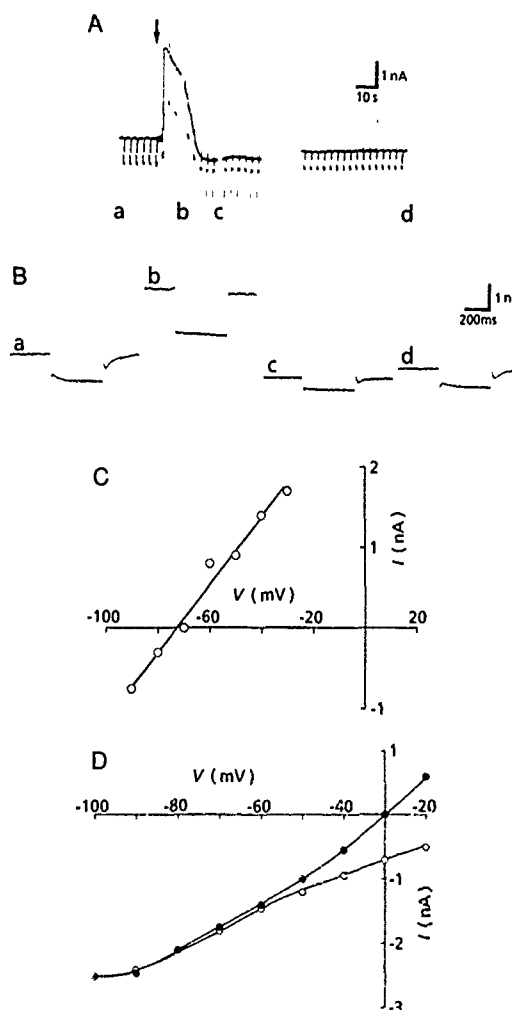
Fig 3 ACh response in m1 transformed NG108-15 cells A Whole-cell currents activated by applying 3 µl of a 0 1 mM ACh solution at the time indicated by the arrow were recorded under voltage clamp at a holding potential of -30 mV. Inward current is downward. Repetitive downward deflections are current transients produced by hyperpolarizing steps of 20 mV for 400 ms applied every 3 s to measure input conductance An interval of ~5 min separates the trace into two parts, and the trace is interrupted by brief periods (~1 s) of faster recording. The recorder saturated during the outward current response. B Expanded records of current transients obtained at the times indicated in A a, before applying ACh, b, during the ACh-induced outward current, c, during the subsequent inward current, d after partial recovery C. Effect of changing clamp holding potential on the ACh induced outward current ACh-activated currents were recorded under voltage clamp at different holding potentials D I-V relationships obtained before applying ACh (filled circles) and during the ACh-induced sustained inward current (open circles). The current was measured as the displacement from the original holding current at the end of incrementing 400 ms voltage steps from a holding potential of -30 mV. From Ref. 19.

A

B

C

D
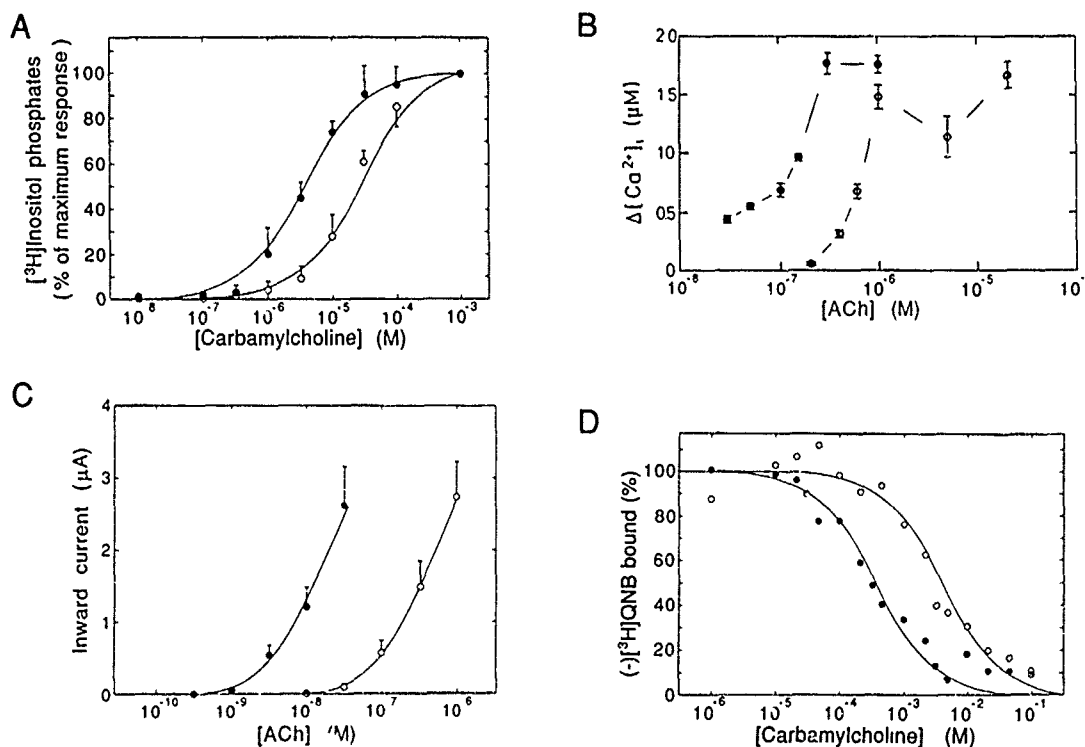
Fig 4 Different agonist sensitivities and binding affinities of m1 and m3 A Dose–response curves for the formation of total [³H]inositol phosphates in transformed NG108-15 cells with porcine m1 (open circles) or porcine m3 (filled circles) Data are expressed as percentage of a maximum response obtained with 1 mM carbamylcholine Each point represents the mean±SD of 3 or 4 experiments B Dose–response curves for ACh-induced intracellular Ca²⁺ release in transformed NG108-15 cells with porcine m1 (open circles) or porcine m3 (filled circles). Each point represents the mean±SE of 7–25 measure-

ments C Dose–response curves for ACh-activated peak inward currents in Xenopus oocytes injected with the mRNA specific for porcine m1 (open circles) or porcine m3 (filled circles). Each point represents the mean±SD of measurements on 10 oocytes at –70 mV membrane potential D Effect of carbamylcholine on (–)[³H]QNB binding in extracts from Xenopus oocytes injected with the mRNA specific for porcine m1 (open circles) or porcine m3 (filled circles) Data are from 3 experiments From Ref. 14 (A, C, D) and Ref 20 (B)

chimaeric mAChR MC2, MC8 or MC10 was oscillatory in nature and was almost completely abolished by intracellular injection of EGTA. On the other hand, a typical ACh response mediated by the chimaeric mAChR MC4 or MC9 comprised an initial smooth inward current which was virtually unaffected by intracellular injection of EGTA, followed by an oscillatory component which disappeared after this treatment. Thus, MC2, MC8 and MC10, in which the putative cytoplasmic portion between the proposed transmembrane segments V and VI is derived from m1, mediate an ACh response similar to that mediated by m1[5,13]. In contrast, MC4 and MC9, which share this portion with m2, elicit an ACh response similar to that induced by m2[13].

Figure 5B shows the antagonist-binding properties of the chimaeric mAChRs in comparison with those of m1 and m2. MC9 exhibited a high affinity for pirenzepine (K_d= 24 nM) similar to that of m1 (K_d= 10–18 nM) and a

low affinity for AF-DX 116 (K_d= 4.1 µM) similar to that of m1 (K_d= 1.8–2.9 µM). In contrast, MC10 showed a low affinity for pirenzepine (K_d= 0.76 µM) similar to that of m2 (K_d= 0.50–0.75 µM) and a high affinity for AF-DX 116 (K_d= 0.57 µM) similar to that of m2 (K_d= 0.36–0.79 µM). These results indicate that the region comprising mostly the putative cytoplasmic portion between the proposed transmembrane segments V and VI is not involved in binding to the selective antagonists. On the other hand, MC4 and MC8 showed intermediate affinity for pirenzepine (K_d= 0.072–0.16 µM) and an intermediate or high affinity for AF-DX 116 (K_d= 0.73–1.1 µM). These results may indicate that both a region containing the putative transmembrane segments I–V and a region containing the putative transmembrane segments VI and VII are involved in antagonist binding.

Our results lead to the conclusion that the region of the mAChR molecules comprising the carboxy-ter-

minal third of the proposed transmembrane segment V and the following putative cytoplasmic portion before the proposed transmembrane segment VI contains a determinant of selective coupling with different effector systems. The amino acid sequence of the putative cytoplasmic portion between the proposed transmembrane segments V and VI is divergent among the mAChR subtypes[5-12,31]. However, some sequence homology is noted in this portion between m1 and m3 as well as between m2 and m4, particularly in the vicinity of segments V and VI. m1 and m3 are coupled with the same effector systems as are m2 and m4. Thus it seems reasonable to assume that a region adjacent to the carboxy-terminus of segment V and a region adjacent to the amino-terminus of segment VI are responsible for selective coupling of mAChR subtypes with different effector systems, probably through interaction with distinct G proteins.

Recent studies with chimaeras between the β_2- and α_2-adrenergic receptors have shown that the specificity for coupling with the stimulatory G protein lies within a

region extending fror the amino-terminus of segment V to the carboxy-terminus of segment VI[32]. Furthermore, a single amino acid substitution in the portion between segments V and VI of rhodopsin has been shown to prevent activation of transducin[33]. Thus, the conclusion that a region mostly comprising the putative cytoplasmic portion between segments V and VI is involved in selective effector coupling may be generally valid for G protein-coupled receptors.

Conclusions

Cloning and sequence analysis of cDNAs or genomic DNAs have revealed the existence of multiple mAChR gene products. The antagonist-binding properties of the individual mAChR species expressed from the cloned DNAs, together with the differential tissue distribution of the mRNAs encoding them, indicate that the mAChR heterogeneity in tissues with respect to antagonist binding can be accounted for by the presence of distinct mAChR species by themselves or in various combinations. The agonist-induced responses

A

B
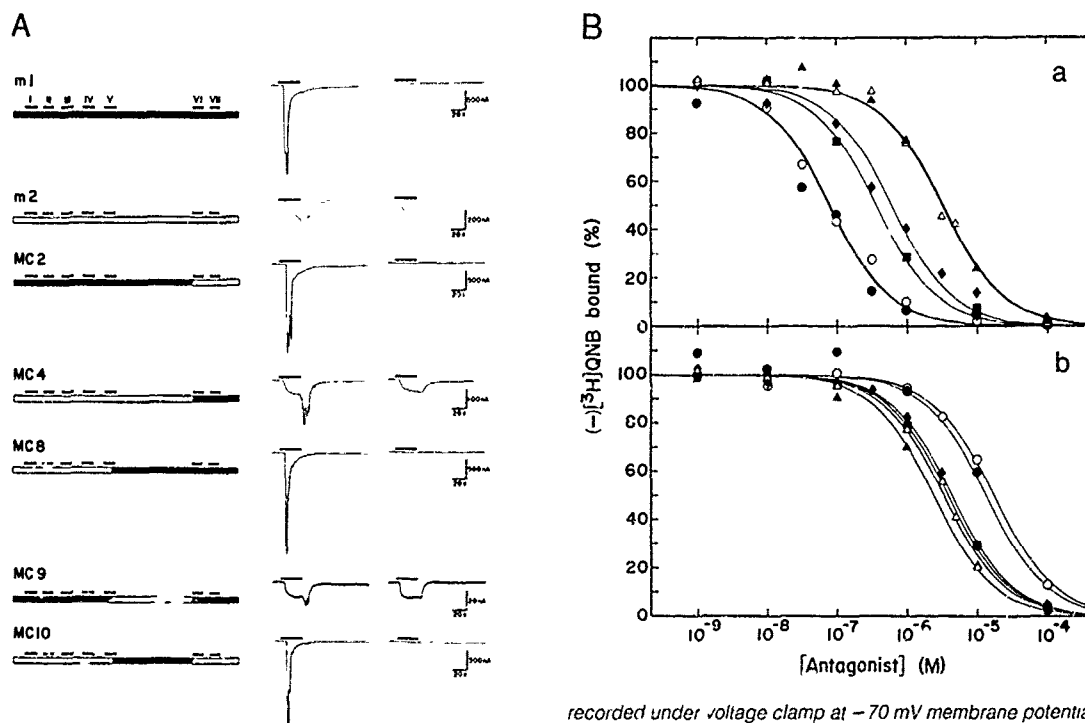
Fig 5 Functional properties of chimaeric mAChRs composed of porcine m1 and m2 sequences A Diagrammatic representation of the structures of chimaeric mAChRs (left half) and ACh responses in Xenopus oocytes injected with the respective chimaeric mRNAs (right half) Sequences of m1 and m2 are indicated by filled and open boxes, respectively, and the putative transmembrane segments I–VII are aligned. Whole-cell currents activated by bath application of 1 μM ACh were recorded under voltage clamp at −70 mV membrane potential in Ringer's solution before (left traces) and after intracellular injection of EGTA (right traces) Inward current is downward. The duration of ACh application is indicated by bars without taking into account the dead-space time in the perfusion system (– 6 s) B Effects of pirenzepine (a) and AF-DX 116 (b) on (−)[³H]QNB binding in extracts from Xenopus oocytes injected with the mRNA specific for m1 (open circles), m2 (open triangles), MC4 (filled diamonds), MC8 (filled squares), MC9 (filled circles) or MC10 (filled triangles) Data are from 3–5 experiments. From Ref. 30

in *Xenopus* oocytes and NG108-15 neuroblastoma-glioma hybrid cells expressing the individual mAChR species provide evidence that the molecularly defined mAChR subtypes are selectively coupled with different effector systems, albeit not exclusively. m1 and m3 are coupled with the same effector systems, but they have different sensitivities to and different binding affinities for agonist. Functional analysis of chimaeric receptors with different combinations of m1 and m2 indicates that a region mostly comprising the putative cytoplasmic portion between the proposed transmembrane segments V and VI contains a determinant of selective coupling with different effector systems.

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New subtypes of muscarinic acetylcholine receptors

Tom I. Bonner

Human and rat genes and/or cDNAs for five different mAChRs have been cloned. The m1, m2 and m3 receptors correspond most closely to the pharmacologically defined M_1, M_2 and M_3 receptors and are expressed in both brain and peripheral tissues. The m4 and m5 receptors are previously unrecognized pharmacological subtypes whose mRNAs are found predominantly in brain. Other less related but uncharacterized genes could represent additional subtypes. The properties of the five receptors and their genes are reviewed and their implications for future research are discussed.

Five different muscarinic receptors have been cloned from both rat and human[1-4], three of which have also been cloned from pig[5-8] and one from mouse[9]. The first two were cloned from pig brain (m1) and heart (m2) using probes based on the amino sequence of purified receptor protein[5-7] and the remainder have been isolated by low stringency hybridization using DNA sequences derived from either m1 or m2 clones as probes. All the muscarinic receptors have the structure composed of seven transmembrane regions usually connected by short extracellular or cytoplasmic segments that we have come to expect for G protein-coupled receptors. One exception is that the muscarinic receptors have a large third cytoplasmic loop of 157–203 amino acids whereas most other receptors of this class have much shorter third cytoplasmic domains. Comparison of the amino acid sequences of the different subtypes of muscarinic receptor indicates that there is little conservation in the amino terminal extracellular domain except for the presence of two or three potential glycosylation sites and that this lack of conservation extends to the middle of the first transmembrane domain. The other major region of sequence difference is the third cytoplasmic domain where there is little, if any, sequence identity among all the receptors although the first and last 20 amino acids are partially conserved among some of the subtypes in a manner which correlates with their second messenger preferences. The remainder of the molecule shows a high degree of sequence conservation with approximately 50% sequence identity (145 amino acids) concentrated primarily in the transmembrane domains and including approximately 40 amino acids which are highly conserved among all the cloned G protein-coupled receptors. This pattern of sequence conservation on an intermediate evolutionary time scale is also reflected on a shorter time scale in the comparison of individual subtypes among mammalian species and is consistent with the hypothesis that the ligand binding site is formed by the transmembrane domains.

Gene structure and chromosomal distribution

Strikingly, the genes for muscarinic receptors as well as several other, but not all, G protein-coupled recep-

tors lack introns in their coding sequences. This observation, first made by comparing rat m1 and m3 and pig m2 cDNAs to their genes[1,7], has made possible the easy cloning of the complete coding sequences of the m4 and m5 receptors from genomic libraries. However, unlike the β_2-adrenergic receptor which appears to have no introns, comparison of cDNA sequences with gene sequences for the m1, m2, m3 and m5 receptors indicates that, while there are no introns in the 3' untranslated sequences, there is at least one intron located less than 100 bases 5' of the initiation codon. Although there has been no direct confirmation, there is a probable splice site in the m4 gene located 29 bases 5' of its initiation codon. The biological significance of the lack of introns in the coding sequence is unclear but some correlation may become apparent as the genes of more receptors of this class are characterized.

We have examined the structure of the m1 gene in more detail by comparing the sequences of the rat and human m1 genes to that of the porcine m1 cDNA sequence since it is approximately the same size as the 3.0 kb mRNA observed in rats. Such a comparison identifies a cluster, extending over 2 kb, of three exons of 90–170 bases each in both the human and rat genes which is separated from the coding exon by an intron of approximately 10 kb. The human exons have 65–91% sequence identity with the porcine cDNA and are immediately preceded by about 300 bases of sequence which is well conserved (80%) in the rat gene. Although this region has yet to be identified as the promoter region by mapping the 5' end of the mRNA, we assume that it will prove to be so. Peralta *et al.* indicate that there are similar additional exons in m2 gene[7]. However, in the case of both the m2 and m3 genes the reported cDNA sequences are 1–3 kb shorter than the 5–6 kb and 4.5 kb mRNA sizes observed in rat. Therefore the characterization of the promoter regions may involve considerable effort. For the m4 gene for which the available cDNA sequences are 600–700 bases shorter than the 3.3 kb mRNA size in rats, we have begun to look for the promoter region by mapping conserved regions, as judged by cross hybridization, between the rat and human genes and have found such a region approximately 10 kb 5' of the coding exon for which sequence analysis and mRNA protection assays should provide an identification. A direct measurement of the size of the m5 mRNA has not been reported

but the recent cloning of a 3' truncated rat m5 cDNA establishes that there are at least 516 bases of 5' untranslated sequence not found in the coding exon[10]. Coupled with our sequence of the 3' untranslated regions of the m5 genes, we can estimate that the mRNA must be at least 3 kb.

In addition to the usual reasons for wanting to know the chromosomal localization of a family of human genes, such as whether they are clustered on a chromosome or dispersed throughout the genome, there is a special reason for wanting to know the chromosomal location of the mAChR genes relating to the cholinergic hypothesis of depression[11]. According to this popular hypothesis, a hypersensitive cholinergic system could represent a predisposing factor for depression. Since predisposing genes for manic depression have been mapped to chromosomes 11 and X in different pedigrees, knowing the location of the mAChR genes would allow one to consider whether they are serious candidate genes for either pedigree. From analysis of somatic cell hybrids as well as chromosomal *in-situ* hybridization we have found that the m2, m3 and m5 genes map to the tips of chromosomes 7(7q35–36), 1(1q41–44) and 15(15q26), respectively (T.I Bonner, W.S. Modi and S.J. O'Brien, unpublished results). The m1 and m4 genes both map to chromosome 11 with m1 being located near the centromere and m4 being somewhere on the short arm. Since the predisposing gene in the Amish pedigree maps near the H-*ras* and insulin genes at the tip of the short arm of chromosome 11, the m4 gene could be marginally considered a candidate gene. Clearly better mapping data are required before it can be considered a serious candidate.

Are there more mAChR genes?

Analysis of genomic blots can provide clues to such questions and in the case of the muscarinic receptors is simplified by the fact that the genes lack introns in their coding sequences. Ideally one would like to use a probe containing as much sequence as possible which is highly conserved among mAChR genes with as little as possible of sequence which is not. As we originally reported, using such a probe containing transmembrane regions 2–5 of the rat m1 cDNA under hybridization conditions which should detect genes with about 75% sequence identity, one can detect 5–6 different bands in human DNA and 9–10 in rat. In each case a single band was ascribable to the m1, m2, m3 and m4 genes[1]. This observation provided the motivation that led to the cloning of the m5 genes. Since the existence of more bands in rat than in human suggested that they might be evolutionarily new and thus possibly pseudogenes and because the human genes are of greater interest, we have focused on the human bands. As we reported, the m5 gene accounts for only one band in *Pst*I digests of human DNA implying that there is at least one more related human gene[2]. The strength of the hybridization signal suggested that it might be more closely related to the m1 gene than some of the other mAChR genes are and thus quite possibly another mAChR gene. However, using a nearly identical probe from the rat m3 cDNA, we detect only the

five known genes so that the character of the sixth gene is unclear. Perhaps it is a pseudogene of the m1 receptor. Such genomic blot analysis does not exclude the possibility that there may be another muscarinic receptor gene which is less related than the m1–m5 genes are to each other, but at this point there is no evidence on which to base an expectation that there will be more subtypes.

Why are there so many receptor subtypes?

This is becoming a common question as cloning of receptors reveals the existence of unanticipated multiple subtypes for a variety of other receptors such as thyroid hormone receptors, neural nicotinic acetylcholine receptors and GABA$_A$ receptors. In general one would expect that the subtypes would differ in their functional properties or in their regulation. The regulatory differences could be either at the transcriptional level through different promoters leading to tissue or developmental specificity, or at the post-transcriptional level through differences in sequence which serve as the sites of regulation through mechanisms such as phosphorylation. An example combining both functional and regulatory factors is the muscular nicotinic receptor which has an embryonic form which differs from the adult form in its channel properties. For the muscarinic receptors there is no evidence for a developmentally specific subtype but there is some degree of tissue specificity and there are at least two functionally distinct classes.

Distribution of receptor mRNAs

Northern blot analysis and *in-situ* hybridization to various tissues has provided considerable data on the tissue specificity of subtype expression. Northern blots of peripheral tissues[12] indicate that the heart expresses only m2 mRNA but that glandular tissues such as lacrimal, parotid and submandibular glands express both m1 and m3 mRNAs. Smooth muscle tissues such as intestines, trachea and urinary bladder express both m2 and m3 mRNA. The high resolution of *in-situ* hybridization[13,14] indicates that m1, m3 and m4 mRNAs are abundantly and broadly expressed in rat brain. Their distributions are largely overlapping but there are differences in their relative abundance in areas such as hippocampus, striatum and dentate gyrus. Except for high levels in a few areas such as the diagonal band and the medial septum, m2 mRNA is expressed widely but at much lower levels[14]. It has only recently become apparent that m5 mRNA is expressed in brain[10] since its abundance is quite low[14]. Although the results could be biased by sensitivity problems, it appears that m5 mRNA is expressed generally in the same regions as m1, m3 and m4 mRNAs but with a more restricted distribution. The mRNA distributions cannot be simply equated with receptor distributions since the mRNAs are localized in cell bodies whereas for neurons the receptors reside on the neuronal projections. Eventually subtype-specific antibodies should allow mapping of the actual receptor distributions. For the present, however, it appears that tissues containing only a single subtype of receptor will be rare.
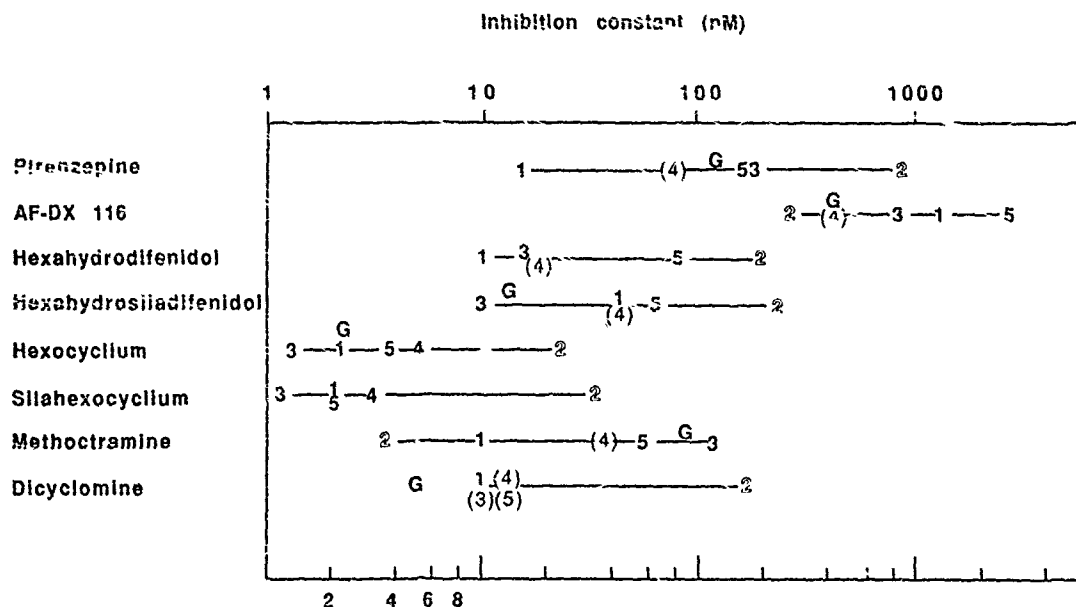
Inhibition constant (nM)

Fig 1 Graphical representation of antagonist binding properties of muscarinic receptor subtypes[20] The numbers 1–5 represent the five cloned subtypes, G represents parallel data on membranes from rat submaxillary gland Values in parentheses are approximations since the Hill coefficients for these data points differed significantly from unity The inhibition constant of AF-DX 116 for the m2 receptor has been corrected for a miscalculation in the conversion of IC50 to K_i

Functional responses of cloned receptor subtypes

Most of what we know about the functional properties of the individual subtypes comes from expressing the clones in cells, such as *Xenopus* oocytes or various mammalian cell lines, which do not express endogenous muscarinic receptors. Since the action of the receptor depends on various components of the second messenger systems as well as their targets, such cells may not contain all the components necessary to elaborate all the potential responses. Nevertheless, such cells offer two major advantages which have allowed a functional distinction to be made among the five receptors. First, the cells which have not had the clones introduced for expression provide an unambiguous control for the specificity of the response. Second, by expressing each of the receptors in the same host cell, one assures, at least if the receptors are expressed at comparable levels, that the receptors all have the same cellular environment so that differences in responses are intrinsic to the subtypes. As recently summarized[15-17], the functional responses of the subtypes divide them into two classes with the odd numbered subtypes in one class and the even numbered in the other, a separation which is also evident in the overall sequence relatedness of the subtypes.

The m1, m3 and m5 receptors stimulate the metabolism of inositolphosphates generally through a pertussis toxin-insensitive G protein and also stimulate the release of arachidonic acid. The release of arachidonic acid is independent of the inositolphosphate stimulation since phorbol esters inhibit the inositolphosphate response but stimulate the arachidonic acid response[18].

This class of receptors has also been observed to cause an increase in cAMP levels. The latter effect has recently been shown for the m1 receptor to be a secondary effect of inositolphosphate stimulation which results in release of intracellular calcium and stimulation of calmodulin-dependent adenylate cyclase (C.C. Felder, R.Y. Kanterman, A.L. Ma and J. Axelrod, *J. Biol. Chem.*, in press). Release of intracellular calcium is also responsible for the opening of Ca^{2+}-dependent potassium channels in transfected mammalian cells.

The m2 and m4 receptors in the same cells cause a decrease of cAMP through a pertussis toxin-sensitive G protein. While they have not shown any electrophysiological response in transfected mammalian cells, these receptors do cause the stimulation of calcium-independent currents in *Xenopus* oocytes.

These mutually exclusive responses parallel the results observed with the endogenous receptors of the 132N1 and NG-108 cell lines[19] which express m3 and m4 mRNAs, respectively. However, there is evidence that the m2 and m4 receptors weakly stimulate an inositolphosphate response at least when there are high numbers of receptors per cell. A likely explanation is that the receptors are not absolutely selective in their coupling to G proteins and that at high levels of receptor enough of a less favorably coupled G protein interacts with the receptor to mediate a response.

Ligand binding properties of the receptor subtypes

Since most tissues can be expected to contain more than one mAChR subtype, the only practical way to sort out the subtype specificities of various ligands may

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be to use the cell lines expressing individual cloned receptors. A number of selective muscarinic antagonists have been tested on such cell lines[20] and the results are summarized in Fig. 1. From these results it should be clear that the m1, m2 and m3 clones correspond most closely to the M_1, M_2 and M_3 pharmacological subtypes while the m4 and m5 have profiles different from the previously described pharmacological subtypes. Curiously, introduction of a silicon atom into hexahydrodifenidol shifts affinities of m1 and m4 relative to all the others even though m1 and m4 are relatively dissimilar in their sequences. Moreover, introduction of the silicon atom into hexocyclium has little effect, shifting m5 and m4 slightly relative to others

It is also clear that these antagonists differentiate m2 to varying degrees from all the other subtypes. In general, however, there is relatively little selectivity among the subtypes which may simply be a reflection of the high degree of relatedness among the five receptors. Nevertheless some pairs, such as m2 and m4, which are closely related in sequence show substantial differences in inhibition constants for some antagonists.

The agonist binding properties have so far received less attention, at least in part because their affinities depend on coupling to G proteins. Even though it is clear that the receptors can all be coupled to G proteins in the artificial environments that have been used to express the cloned receptors, it is not obvious that in their normal environment they could not couple to a G protein such as G_0, which is presumably absent in these cells, to produce a different agonist affinity.

Functional domains of the receptors

Our models for the structure of the binding sites of muscarinic receptors come primarily from the extrapolation of results with the β-adrenergic receptor and rhodopsin which indicate that the binding site is located within the transmembrane domains. Since the m2 and m4 receptors differ in their transmembrane domains by only 17 amino acids (with 11 of those differences in the first or sixth transmembrane regions) and have affinities for pirenzepine which differ by about twentyfold, we have sought in our initial attempts to define the ligand binding site to localize the site responsible for this difference (R. Lahti, N.J. Buckley, A.C. Young, M R. Brann and T.I. Bonner, unpublished results). To do this we have examined chimeras of human m2 and rat m4 receptors using conserved *PvuII* and *SmaI* sites located at the beginning of the fourth transmembrane region and immediately before the sixth transmembrane region as the points of fusion. These two points divide the molecules roughly into thirds containing 9, 3, and 5 amino acid changes in the transmembrane regions. Substitution of either the last third or the last two thirds of the m4 receptor into the m2 receptor leads to a four- or fivefold increase in the affinity for pirenzepine while substitution of the middle third has little or no effect. Although the small differences in affinity are difficult to measure accurately, these results seem to indicate that there are regions in the first and last thirds of the molecule which have roughly equal effects on the pirenzepine affinity.

It will therefore be difficult to use this approach to localize even specific transmembrane regions involved in determining antagonist affinity without a more selective ligand.

There is now evidence based on chimeric receptors for both the adrenergic and muscarinic receptors that the third cytoplasmic domain of the receptors is sufficient to determine the second messenger coupling[21,22]. If one compares the sequences of this domain of the muscarinic receptors with those of other receptors which are known to couple to inositolphosphate metabolism or cyclase inhibition there is no obvious pattern of sequence conservation. However among the muscarinic receptors the first and last 15–20 amino acids of this domain, while not well conserved among all the receptors, do have greater than 50% sequence identity either among the odd numbered receptors which couple preferentially to inositolphosphate metabolism or between the even numbered ones which couple preferentially to inhibition of adenylate cyclase. Since the analogous regions of the $β_2$-adrenergic receptor have been shown by deletion analysis to be necessary for second messenger coupling[23], it seems likely that one or the other or both of these short segments may confer second messenger specificity. To test this hypothesis we have begun to examine chimeras of the human m2 and rat m3 receptors by determination of their inositolphosphate response when transiently expressed in COS cells[24]. In this system, as has been seen before for high levels of receptor expression in CHO cells[7], the m2 receptor gives a modest inositolphosphate response with an ED50 at least tenfold greater than the m3 receptor. Substitution of the m3 third cytoplasmic domain into the m2 receptor converts the response to one comparable to that of the m3 receptor. Furthermore substitution of the first 17 amino acids from the m3 domain into the m2 receptor produces an inositolphosphate response comparable in magnitude to that of the m3 receptor but requiring a tenfold higher agonist dose These results need to be extended to include substitutions of the last 20 amino acids of the third cytoplasmic domain and assays of cAMP responses to assure that the appropriate reciprocal effects are seen. However, based on the present results, it appears that the first 17 amino acids confer specificity of coupling but that something else, such as the last 20 amino acids, is necessary for efficient transmission of conformational change from ligand binding site to G protein. Given the nearly universal conservation of the beginning of the second cytoplasmic domain among all G protein-coupled receptors, it is probable that this domain also plays a role in G protein coupling, perhaps as a site of more general interaction with all G proteins.

Questions for the future

Which G proteins do the receptors couple to?

Does a single receptor couple to more than one G protein depending on cellular environment? Is there a hierarchy of G-protein affinities? These are not trivial experiments to do since the systems used to date to express the cloned subtypes all contain G proteins capable of coupling to the receptors.

Are the odd numbered and the even numbered receptors really functionally homogeneous?

The cloned receptors have so far been tested in only rather unnatural host cells which may very well lack the ability to provide some of the responses available to the receptors in the cells in which they are normally expressed. A clear example of this situation is provided by the use of NG-108 cells, which normally express m4 receptors, for expression of cloned m1–m4 receptors[25]. The m1 and m3 receptors but not m2 and m4 receptors were shown to inhibit the M-current, a response not available in A9 fibroblasts. More such cells will have to be used to explore the full repertoire of muscarinic responses to see if the receptors are as functionally interchangeable as they now appear to be.

Are the receptors differently regulated at the transcriptional or post-translational levels?

The tools are now available to examine mRNA changes during development or as a result of various manipulations of animals or cell lines to see whether there are regulatory differences other than those which specify in which cells the receptors are expressed At the post-transcriptional level, it is commonly thought that the activity of the receptors could be regulated by phosphorylation. A likely place for the phosphorylation sites would be in the third cytoplasmic domain most of which is quite different for the different subtypes. Differential phosphorylation might therefore allow for substantial differences in desensitization properties of the subtypes.

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Functional diversity of muscarinic receptor subtypes in cellular signal transduction and growth

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The regulation of cellular signal transduction and growth by four human muscarinic acetylcholine receptor (mAChR) subtypes has been studied comparatively. The four mAChRs fall into two functional sub-groups, based on their primary effects on second messenger formation, two of the receptors strongly inhibit adenylyl cyclase activity, whereas the other two strongly stimulate PI hydrolysis. Studies on mAChR regulation of two cellular events involved in cellular growth regulation, the transcription of proto-oncogene c-fos and DNA synthesis, indicate that these events are efficiently activated by those mAChRs which couple primarily to phospholipase C.

The pharmacological complexity of mAChRs has long indicated not only the existence of multiple receptor subtypes, but also that each subtype is distinct in the cellular and physiological actions of acetylcholine which it carries out[1]. The molecular cloning of five mAChR genes of humans and other mammals, by our group and others[2-8], has demonstrated the molecular basis of mAChR subtype diversity and has allowed the systematic study of their functions in cells normally lacking mAChRs, but apparently possessing many of the cellular signal transduction systems with which these receptors can interact. Here we review our efforts to date to characterize the distinct properties of four human mAChRs, with particular attention to their specific roles in cellular signal transduction and growth. We refer to these four human mAChR subtypes by our original designation, as well as the nomenclature used in this supplement, to avoid any confusion [hence M1 ≡ m1, M2 ≡ m2, M3 ≡ m4 and M4 ≡ m3].

The four human muscarinic receptor subtypes differentially regulate levels of cyclic AMP and inositol phosphates

Regulation of adenylyl cyclase and phospholipase C by mAChRs has been well documented[1]. Experiments with tissue homogenates and cell lines have suggested that these second-messenger-generating enzymes are regulated differentially by different mAChR subtypes[1], however the complex pattern of mAChR subtype expression in different cells and tissues[5-10] has limited the interpretation of such studies. To systematically address this question, we have developed mammalian cell lines stably expressing each mAChR gene product

by transfecting cell lines essentially lacking endogenous mAChRs. We first demonstrated the feasibility of this approach with the porcine atrial mAChR gene, recombinant m2 (PM2) receptors, expressed in Chinese hamster ovary (CHO) cells, were shown to bind muscarinic ligands with affinities comparable to porcine atrial mAChRs, couple to endogenous G proteins, and thus regulate the activity of both adenylyl cyclase and phospholipase C in these cells[11]. We have since investigated the ability of four human mAChR subtypes to regulate second messenger formation in transfected human kidney cell lines expressing comparable levels of each receptor[12]. Figure 1 summarizes the effect of each mAChR subtype on cAMP levels (shown as a carbachol dose-response) and phosphoinositide (PI) hydrolysis (shown as the kinetics of accumulation of inositol phosphates at saturating agonist concentration). These findings indicate greater functional similarity between the Hm1 (HM1) and Hm3 (HM4) receptors, and Hm2 (HM2) and Hm4 (HM3) receptors. In Hm2- (HM2)- and Hm4- (HM3)-expressing cells, carbachol efficiently inhibits adenylyl cyclase activity (forskolin-stimulated cAMP accumulation is decreased by 65–75%), but weakly, although significantly, activates PI hydrolysis. In contrast, carbachol strongly stimulates PI hydrolysis, but does not inhibit adenylyl cyclase activity in Hm1- (HM1)- and Hm3- (HM4)-expressing cells, instead, cAMP levels increase substantially over forskolin-induced levels (7.5–10-fold) at high carbachol concentrations. The elevated cAMP levels thus observed may result from 'cross talk' between PI hydrolysis and cAMP formation, it is known that the PI hydrolysis products inositol tris-phosphate (IP_3) and diacylglycerol can increase the levels of cytosolic calcium and of protein kinase C activity and hence activate adenylyl cyclase[13-16]. It is therefore possible that adenylyl cyclase is activated by the Hm1 (HM1) and Hm3 (HM4) receptors indirectly as a result of their efficient stimulation of PI hydrolysis. This notion is supported by the observation that the carbachol EC50 values for the stimulation of cAMP levels and PI hydrolysis are comparable[12].

We conclude from these studies that mAChR subtypes indeed differ in their ability to regulate second

messenger formation. A given mAChR subtype can regulate more than one effector enzyme, but can be more efficient in activating one response than another, both in the number of receptors per cell and in the agonist concentration required to give an effect. We suggest that the specific response of a given cell to acetylcholine will depend on the particular mAChR subtypes present and their relative abundance, as well as on the concentration of acetylcholine. It is of interest to note that the subtypes we find to be functionally similar are also more similar in amino acid sequence, particularly in the cytoplasmic loop connecting the fifth and sixth transmembrane segments of the receptor[5]

Distinct G proteins selectively couple different mAChR subtypes to PI hydrolysis in the same cell

At least two types of G protein capable of coupling receptors to phospholipase C (termed G_p) have been described. These G_p types can be distinguished by their sensitivity to ADP-ribosylation by pertussis toxin (PTX), which leads to their uncoupling from receptors[17]. Previous work has shown that PI hydrolysis mediated by mAChRs in different cells and tissues is either PTX-sensitive[11,18] or PTX-insensitive[19-21]. The differential ability of mAChR subtypes to stimulate PI hydrolysis in the same cell prompted us to ask whether different G_p proteins are responsible for the selective coupling of different mAChRs to phospholipase C. We have addressed this possibility in two ways: first, we have examined the PTX sensitivity of PI hydrolysis in CHO cells, mediated by the four human

mAChRs and other receptors endogenous to these cells; second, we have examined whether the PI hydrolysis responses mediated by different receptors expressed in the same cell are additive.

Figure 2 shows the effects of PTX on the accumulation of IP_3 resulting from activation of PI hydrolysis by Hm1 (HM1), Pm2 (PM2), or Hm3 (HM4) mAChRs, or two endogenous G protein-coupled receptors in CHO cells, the cholecystokinin (CCK) and thrombin receptors (top panels). Three patterns of PTX sensitivity were observed: the response mediated by CCK receptors is not inhibited by PTX (panel A), the Hm1 (HM1) and Hm3 (HM4) receptor responses display partial sensitivity (panels B and C), and the Pm2 (PM2) and thrombin receptor responses are completely inhibited by PTX (panels D and E). These results are most easily explained by a model in which CHO cells possess two types of G_p protein: a PTX-insensitive G_p, which mediates the coupling of CCK receptors to phospholipase C, and a PTX-sensitive G_p, which mediates the coupling of thrombin and Pm2 (PM2) receptors to phospholipase C. The response mediated by Hm1 (HM1) and Hm3 (HM4) may thus be mediated by both G_p proteins, or alternatively, by yet another G_p, which is only partially attenuated by PTX.

Such a model predicts that the PI hydrolysis responses mediated by different pathways (i.e. different G_ps) will be additive under conditions in which a given pathway can be saturated. Our system afforded us the opportunity to test this, as we are able to construct cell lines in which the response mediated by a given mAChR can indeed be saturated, by creating cell
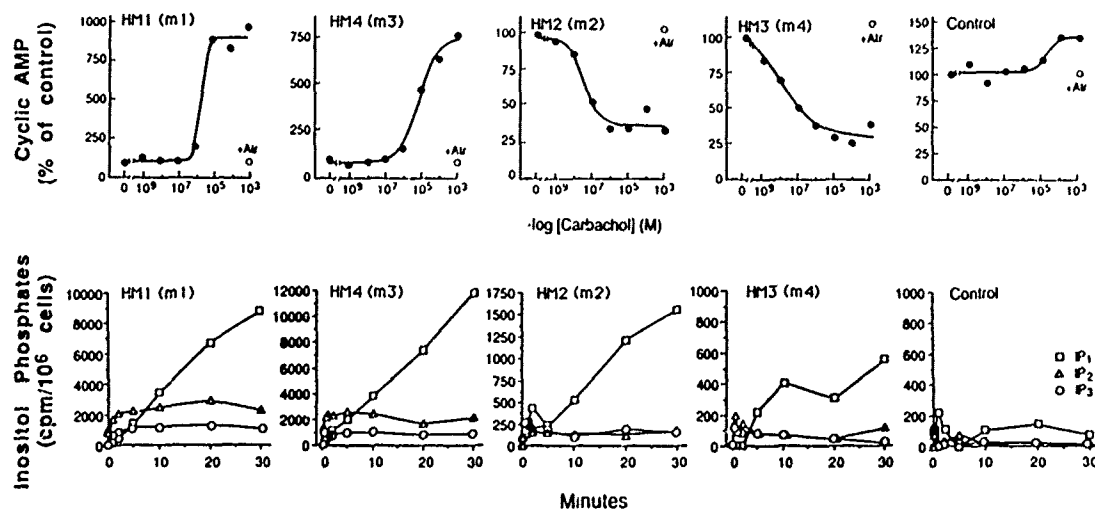
Fig 1 Differential regulation of cAMP formation and PI hydrolysis by human mAChRs in transfected human kidney cell lines. The results depicted are from cell lines expressing 1.4×10^5 Hm1 (HM1), 2.0×10^5 Hm3 (HM4), 1.2×10^5 Hm2 (HM2) and 1.7×10^5 Hm4 (HM3) receptors per cell, or control untransfected cells (300 endogenous mAChRs per cell). Adenylyl cyclase dependent accumulation of cAMP (top panels) was measured in the presence of the phosphodiesterase inhibitor, IBMX (0.2 mM) and the direct adenylyl cyclase activator, forskolin (10 μM). Basal and forskolin-stimulated levels of cAMP were 2.4–4.1 pmol/10^6 cells and 57–270 pmol/10^6 cells, respectively. PI hydrolysis (bottom panels) was analyzed in cells labeled with [^{3}H]myo-inositol, by measuring the accumulation of labeled inositol monophosphate ([^{3}H]IP$_1$) (boxes), inositol bisphosphate ([^{3}H]IP$_2$) (triangles) and inositol trisphosphate ([^{3}H]IP$_3$) (circles) in the presence of 10 mM LiCl, which inhibits dephosphorylation of IP$_1$ (Ref 13) This figure is reproduced with permission from Ref. 12, where complete details on the methodology are given
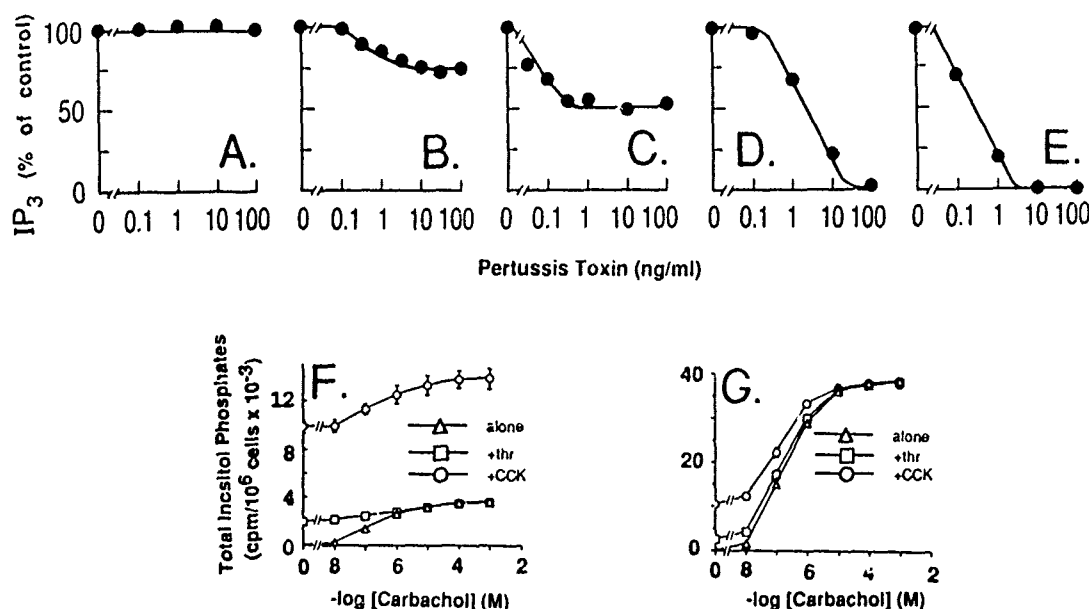
Fig 2 Evidence for distinct G_p-coupled pathways of PI hydrolysis in CHO cells Top panels Effect of pertussis toxin on the activation of PI hydrolysis by various receptors in CHO cells Cells labeled with [³H]myo-inositol were treated with pertussis toxin (6 hours, 37°C) and treated with agonist to elicit maximal stimulation of PI hydrolysis (shown as IP_3 accumulation) Cells are (panels A,E) untransfected cells, (panels B–D) transfected cells expressing (B) 3 8 X 10⁵ Hm1 (HM1) receptors per cell, (C) 1 9 X 10⁵ Hm3 (HM4) receptors per cell, (D) 1 0 X 10⁶ Pm2 (PM2) receptors per cell Agonists are (A) CCK (10 U/ml), (B–D) carbachol (1 mu), (E) thrombin (1 U/ml) 100% levels in cpm/10⁶ cells were 1200±50 (A), 4840±200 (B), 1400±130 (C), 310±20 (D) and 510±30 (E) Bottom panels. Additivity of the PI hydrolysis response mediated by different receptors co-expressed in the same cell Cells expressing levels of Pm2 (PM2) (F) or Hm1 (HM1) (G) receptors sufficient to saturate the PI hydrolysis response to carbachol (1 75 X 10⁶ and 6 0 X 10⁵ receptors per cell, respectively) were labeled with [³H]myo-inositol and treated with various concentrations of carbachol, either alone, or in the presence of saturating concentrations of thrombin (1 U/ml) or CCK (10 U/ml) PI hydrolysis, shown as the accumulation of $IP_1+IP_2+IP_3$, was determined after 30 min of incubation with agonists in the presence of 10 mv LiCl, to minimize differences in accumulation due to kinetic variation This figure is reproduced with permission from Ref 22

lines with high receptor expression levels through gene amplification[11,22]. Thus, the response mediated by a given receptor would be additive to the response mediated by another, assuming the latter response were saturated and coupled by another G_p pathway. This is illustrated in Fig. 2 (bottom panels), for cells expressing spare Pm2 (PM2) receptors (panel F), the PI hydrolysis response to carbachol is not additive with the thrombin-mediated response, but is fully additive with the CCK response, indicating that Pm2 (PM2) and thrombin receptors share a common G_p pathway, whereas CCK receptors use a separate G_p pathway. In contrast, in cells expressing spare Hm1 (HM1) receptors (panel G), the maximal PI response to carbachol is not increased by the presence of either thrombin or CCK, indicating that Hm1 (HM1) receptors are coupled to PI hydrolysis by using both of the distinct pathways used by CCK and thrombin receptors. These results provide strong support for the model suggested earlier for the coupling of PI hydrolysis by two G_p proteins. Thus, in a given cell, individual mAChR subtypes can employ distinct G_p proteins to regulate PI hydrolysis; these G_p proteins are employed selectively by endogenous receptors of CHO cells as well. Significantly, the PI

hydrolysis response mediated by receptors coupled primarily by the PTX-insensitive G_p [Hm1 (HM1), Hm3 (HM4), CCK] is greater than the response stimulated by receptors coupled by the PTX-sensitive G_p [Pm2 (PM2), thrombin] (see Fig. 2 legend). Thus, the principal purpose of selective G_p coupling of PI hydrolysis in a given cell may be to establish different levels of the response in an agonist-specific fashion.

Differential regulation of c-*fos* mRNA expression by mAChR subtypes

Several neurotransmitter receptors, including mAChRs, have been shown to induce transcription of the proto-oncogene c-*fos*[23,24]. The c-*fos* gene product is a nuclear phosphoprotein which may be involved in the control of cell growth and differentiation, by coupling transient changes in extracellular conditions to long-term cellular alterations[25], and is thought to be affected by PI hydrolysis. To determine whether c-*fos* mRNA is induced differentially by mAChR subtypes, we compared the response mediated in transfected CHO cells by Hm1 (HM1) and Hm2 (HM2), which regulate PI hydrolysis by the two distinct pathways described above (Fig. 3). An analysis of c-*fos* mRNA levels by

RNA blot hybridization revealed that carbachol induces a significant increase in c-*fos* transcription in both Hm1-(HM1)- and Hm2- (HM2)-expressing cells. Each response is mAChR-dependent, as it is completely blocked by atropine, and each displays the transient kinetics typical of c-*fos* mRNA induction by many agents[25]. An important difference between the Hm1 (HM1) and Hm2 (HM2) response is apparent in that c-*fos* transcription induced by Hm1 (HM1) is substantially greater and more prolonged, even at a much lower level (20-fold less) of Hm1 (HM1) receptor number per cell. Furthermore, the effect of PTX on c-*fos* transcription is also different; the induction by Hm1 (HM1) is only slightly inhibited, whereas the induction by Hm2 (HM2) is completely abolished by the toxin. Thus, the differential induction of c-*fos* mRNA by Hm1 (HM1) and Hm2 (HM2) strikingly resembles their differential activation of PI hydrolysis, both in the magnitude of response and its sensitivity to PTX.

Differential regulation of DNA synthesis in brain-derived cells by mAChR subtypes

Several observations by other investigators have led us to hypothesize that mAChRs may regulate DNA synthesis in brain-derived cells. First, PI hydrolysis, which certain mAChRs stimulate very efficiently, has been implicated in the regulation of growth of other cell types by other growth factors and neurotransmitters [13,14,26]. Second, added acetylcholine can stimulate PI hydrolysis in developing brain; this stimulation peaks in the perinatal period[27]. Finally, carbachol can stimulate PI hydrolysis in primary cultures of astrocytes from perinatal brain[28]; astrocytes are a major subset of brain cells which proliferate perinatally[29].

To test this hypothesis, we examined the effect of carbachol on DNA synthesis in primary astrocyte cultures (greater than 90% GFAP-positive cells), taken from rat brain at various times during embryonic and neonatal development[30]. As shown in Fig. 4, following serum-deprivation of such cultures, carbachol elicits a significant increase in DNA synthesis, observed by increased incorporation of [³H]thymidine, which peaks at the day of birth (panel A). This effect is substantial (75% of the level induced by fetal calf serum), and is blocked by atropine, indicating its dependence on mAChRs (panel B). Oxotremorine, a mAChR agonist which does not stimulate PI hydrolysis[31,32], does not affect DNA synthesis; noradrenaline stimulates DNA synthesis by approximately 30%, whereas angiotensin II has no effect (panel B). An analysis of individual, single-cell-derived astrocyte cultures revealed that some clones respond well to carbachol, whereas others respond poorly[30]. Given the dependence of this response on mAChRs, and our previous finding that glial and neuronal cell lines express specific subsets of mAChR subtype mRNAs[10], we reasoned that differences in mAChR expression might explain the variable response to carbachol. To investigate this, we analyzed the effects of carbachol on DNA synthesis in several brain-derived glial and neuronal cell lines which we found to express distinct patterns of mAChR subtype mRNA (panel C). Carbachol stimulated DNA synthesis approximately four-fold in 1321N1 astrocytoma cells, which express m3 (M4) mAChRs[10], and SK-N-SH

neuroblastoma cells, which express m3 (M4) and m2 (M2) mAChRs[10]; in contrast, carbachol did not stimulate DNA synthesis in NG108-15 neuroblastoma X glioma and N1E-115 neuroblastoma cells, which express only m4 (M3) receptors[5,10]. Thus, the ability of these brain-derived cell lines to initiate DNA replication in response to carbachol appears to be due to expression of specific mAChRs [i.e. m3 (M4), but not m4 (M3)], but could also result from other differences between the cells.

To evaluate these possibilities, we examined the ability of carbachol to stimulate DNA synthesis in a single cell type (CHO cells), expressing each of the four human mAChRs individually[30] (Fig. 4, panels D–G). DNA synthesis is stimulated by carbachol significantly in cells expressing each of the four receptor subtypes, however, the response is much greater in cells expressing Hm1 (HM1) or Hm3 (HM4) than cells expressing Hm2 (HM2) or Hm4 (HM3). In each case, the response increases with receptor number, but the number of Hm2 (HM2) and Hm4 (HM3) receptors required to give an effect comparable to that mediated by Hm1 (HM1) or Hm3 (HM4) receptors is markedly higher. These results indicate that mAChR subtypes indeed differ in their
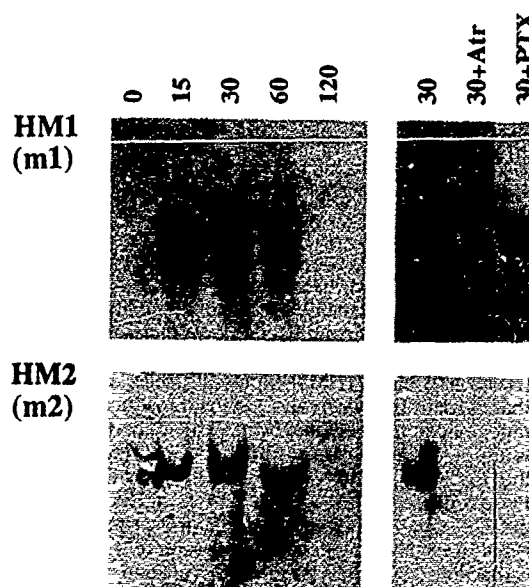
Fig 3. Differential induction of c-fos mRNA levels by mAChR subtypes in transfected CHO cells CHO cell lines expressing Hm1 (HM1) or Hm2 (HM2) (5 X 10⁴ and 1 X 10⁶ receptors per cell, respectively) were treated with carbachol (1 mM) for 0, 15, 30, 60, or 120 min (left hand panels), or for 30 min with either carbachol, carbachol and 10 μM atropine (Atr), or carbachol after a 6 hour treatment with 100 ng/ml pertussis toxin (PTX) (right hand panels). Total RNA was isolated, electrophoresed on agarose gels (15 μg per lane), blot transferred to nylon filters, and hybridized with a [³²P]-labeled v-fos cDNA probe The size of the c-fos transcript detected is 2 2 kb, relative to ribosomal RNA. Rehybridization of the blot with an α-actin probe demonstrated that comparable amounts of total RNA were present in each lane (not shown).
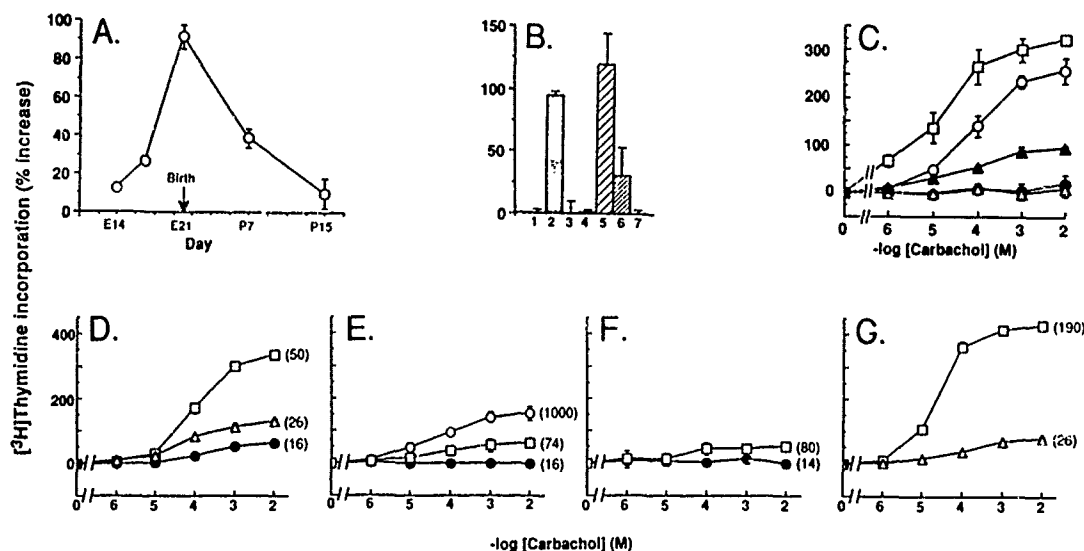
Fig 4. DNA synthesis is activated by carbachol in primary astrocytes, in brain-derived cell lines, and in transfected CHO cells expressing mAChRs Panel A. Primary astrocyte cultures, derived from rat brain at embryonic day (E) 14 to postnatal day (P) 15, were deprived of serum for 48 hours, incubated with carbachol (1 mм) and [^{3}H]thymidine (2 μCi/ml) for 24 hours, and [^{3}H]thymidine incorporation measured Panel B Primary astrocytes derived from E21 rats were deprived of serum and incubated as above with either (1) no added agonist, (2) 1 mм carbachol, (3) 1 mм carbachol and 10 μм atropine, (4) 1 mм oxotremorine, (5) 10% fetal calf serum, (6) 100 μм noradrenaline, (7) 10 μм angiotensin II. Panel C Dose-response analysis of carbachol stimulation of DNA synthesis in primary astrocyte cultures (filled triangles), 1321N1 human astrocytoma cells (open circles), SK-N-SH human neuroblastoma cells (open boxes), NG108-15 mouse neuroblastoma X rat glioma hybrid cells (filled circles), NIE-115 mouse neuroblastoma cells (open triangles). Panels D–G. Transfected CHO cell lines expressing various levels of (D) Hm1, (E) Hm2, (F) Hm4, (G) Hm3 mAChRs (numbers denote 10^{-3} X receptors per cell) were deprived of serum and analyzed for dose response of carbachol-stimulated DNA synthesis. This figure is reproduced with permission from Ref. 30, where complete methodological details are given.

ability to support carbachol-stimulated DNA synthesis in a given cell. Notably, carbachol stimulates both PI hydrolysis and DNA synthesis more efficiently in Hm1-(HM1)- and Hm3- (HM4)-expressing cells than in Hm2-(HM2) and Hm4- (HM3)-expressing cells, suggesting that PI hydrolysis is involved in the transduction of the mitogenic signal mediated by mAChRs. This possibility is supported by several additional observations. First, the carbachol EC50 values for DNA synthesis and PI hydrolysis in CHO cells expressing recombinant mAChRs, brain-derived cell lines, and primary astrocytes are comparable, and quite different from the carbachol EC50 values for inhibition of adenylyl cyclase seen for Hm2 (HM2) and Hm4 (HM3) receptors in CHO cells[30]. Second, in CHO cells expressing Hm1 (HM1), Hm2 (HM2), or Hm3 (HM4) receptors, PTX inhibits carbachol-stimulated DNA synthesis and PI hydrolysis to a similar extent[22,30]. Finally, DNA synthesis in primary astrocytes is not stimulated by oxotremorine, a muscarinic agonist which does not stimulate PI hydrolysis[31,32]. These results thus indicate that mAChR activation of PI hydrolysis, rather than inhibition of adenylyl cyclase, is involved in mitogenic signaling by carbachol.

The age-dependent activation of DNA synthesis by carbachol in neonatal astrocyte cultures indicates that acetylcholine may regulate astroglial cell growth during brain development. Thus, the expression of certain mAChR subtypes in specific astrocytes may function to coordinate the proliferation of these cells with the development of cholinergic neurons.

Conclusion

By studying the properties of recombinant mAChRs expressed individually in heterologous cells, we have begun to dissect the cellular functions of mAChR subtypes. As summarized in Fig. 5, we have shown that mAChR subtypes selectively regulate the formation of different second messenger molecules (e.g. cAMP or IP$_3$ and diacylglycerol). Thus, Hm1 (HM1) and Hm3 (HM4) are primarily coupled to the activation of phospholipase C, whereas Hm2 (HM2) and Hm4 (HM3) are primarily coupled to the inhibition of adenylyl cyclase. Hm2 (HM2) and Hm4 (HM3) are also linked to the activation of phospholipase C. However, this coupling is much less efficient than that mediated by Hm1 (HM1) and Hm3 (HM4). In addition, carbachol strongly stimulates cAMP formation in Hm1- (HM1)- and Hm3- (HM4)-expressing cells. In view of previous evidence for 'cross talk' between the phospholipase C and adenylyl cyclase systems[14-16], the similarity in carbachol EC50 values for cAMP elevation and activation of PI hydrolysis by Hm1 (HM1) and Hm3 (HM4) appears to indicate that cAMP elevation by these

subtypes may result from their efficient coupling to PI hydrolysis. However, it is also possible that the observed cAMP elevation results from a direct coupling of adenylyl cyclase by a stimulatory G protein similar to G_s.

We have shown that mAChR subtypes, as well as other G protein-coupled receptors (such as CCK and thrombin receptors), are selectively coupled to PI hydrolysis by distinct G protein pathways (designated G_p and G_p^* in Fig. 5) in the same cell. These pathways appear to differ in the magnitude of the PI hydrolysis response; the PTX-insensitive G_p found in CHO cells is associated with a substantially greater activation of PI hydrolysis than the PTX-sensitive G_p found in the same cells. In the regulation of adenylyl cyclase, specificity results from the selective coupling of different receptors (or receptor subtypes) to either stimulatory (G_s) or inhibitory (G_i) G proteins. The present findings indicate that specific regulation of phospholipase C activity may be achieved by selective coupling of different receptors (or receptor subtypes) by multiple stimulatory G proteins (G_p, G_p^*), which activate different levels of PI hydrolysis. It is interesting to speculate that selective coupling of these G_p proteins to the multiple phospholipase C gene products recently identified by molecular cloning[33-35] may in turn explain the different magnitudes of PI hydrolysis generated by each G_p pathway.

We have also shown that mAChR subtypes differ significantly in their ability to mediate induction of c-*fos* transcription and DNA synthesis. Beca.. ' vdrolysis appears to be linked to these cellular ev.   c had anticipated that mAChR subtypes which a   ngly coupled to phospholipase C would be imj   ·n

their induction by carbachol. This prediction was confirmed in both cases, and evidence was obtained which indicates that PI hydrolysis may indeed be a major mechanism by which carbachol induces c-*fos* transcription and DNA synthesis. Note that the regulation of second messenger formation and DNA synthesis by recombinant mAChRs in heterologous cell models and by mAChRs expressed in brain-derived cell lines is essentially identical (Ref. 30 and refs cited therein), thus confirming the physiological relevance of the approach we have undertaken.

Muscarinic receptor subtypes provide a striking example of how structural diversity in a signal transduction component can be used to achieve functional specificity in cellular signaling and growth. As the pattern of mAChR subtype expression is highly cell-specific and multiple subtypes are often expressed in a single cell[10], even greater diversity in the response of individual cells to acetylcholine is predicted. It is important to emphasize that the usefulness of the approach we have described will depend upon the particular components of the signal transduction apparatus expressed in the recipient cell type used for *in vivo* 'complementation analysis' Further functional differences between apparently similar subtypes are to be expected, for example, in their ability to regulate ion channels, and the appropriate choice of recipient cells containing such components, or the co-expression of such components by DNA-mediated gene transfer, will be necessary to complete the analysis of functional differences between mAChRs which we have described here.

Fig. 5 Summary of functional differences of human mAChR subtypes in cellular signal transduction and growth G_p and G_p^* PTX-insensitive and -sensitive G proteins, respectively, which couple stimulatory receptors to phospholipase C (PLC) G_i: G protein which couples inhibitory receptors to adenylyl cyclase (AC). DAG diacylglycerol PKC, protein kinase C.

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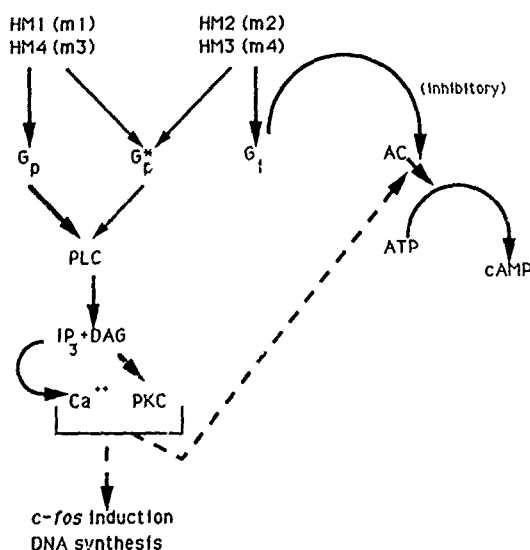21 Chiba, T , Raffoul, K and Yamada, T. (1987) *J. Biol. Chem.* 252, 8467–8469

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Localization and structure of the muscarinic receptor ligand binding site

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A conserved aspartic acid residue in transmembrane helix 3 of the muscarinic acetylcholine receptors is important in binding the headgroup of muscarinic ligands. This acidic amino acid probably points into a relatively hydrophilic cavity whose walls are formed by the amphipathic transmembrane helices of the receptor. Amino acid side chains within this cavity contribute to ligand binding.

From the structural as well as the pharmacological viewpoint, amongst the most interesting and fundamental questions which may be asked about a family of receptors such as the muscarinic receptors (mAChRs) are the following: Where is the ligand binding site located? What are the key interactions which mediate ligand binding? How does the binding of agonists differ from that of antagonists? What are the important structural features of the receptor which enable and transmit the agonist-induced conformational change?

The mAChRs belong to the category of G protein-coupled cationic amine receptors, whose ligands, both agonists and antagonists, incorporate a basic nitrogen atom. About 20 distinct members of the G protein-coupled receptor superfamily have now been cloned and sequenced, including five subtypes of mAChRs (Refs 1–3). These molecules are all thought to conform to a generic structure consisting of seven trans-membrane helices, joined by intra- and extracellular loops of varying lengths, and with a glycosylated extracellular N-terminal sequence and an intracellular C-terminal tail[1]. The muscarinic receptors contain several conserved aspartic acid residues, particularly Asp71, Asp99, Asp105 and Asp122 (m1 mAChR sequence, using the nomenclature recommended on p. V) which are potential participants in binding the cationic headgroups of muscarinic antagonists and agonists.

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Two approaches are in current use to try to identify amino acids in receptor sequences which interact directly with ligand functional groups. The first is to mutate the suspected residues, and then to study the binding and functional properties of the mutant receptor. The second is to design ligand molecules which incorporate chemically-reactive functional groups, to allow them to bind and react, and then to identify the modified amino acids.

Both approaches have their strengths and weaknesses. Briefly, these are that mutation of an amino acid may alter the three-dimensional structure of the receptor in what amounts to an allosteric fashion. A large effect on ligand binding therefore does not necessarily imply a direct binding interaction between the modified amino acid and the ligand. In contrast, whilst the use of chemically-reactive ligand analogues enables one to work on the native, un-mutagenized receptor, reaction may also occur with amino acid side chains outside the binding site. Fortunately, the two approaches are complementary, and where they converge in pinpointing a residue, we may reasonably believe that residue to be important.

We have taken the chemical approach to trying to identify important residues in the muscarinic binding site. Thanks to a tradition of molecular pharmacology dating back four decades to the work of Nickerson on irreversible blockers of the adrenergic receptors, a suitable chemical tool has been at our disposal for a number of years in the form of [³H]propylbenzilylcholine mustard ([³H]PrBCM).

Muscarinic receptors are labelled with great specificity by nanomolar concentrations of [³H]PrBCM[4,5]. The benzilylcholine mustards have two great advan-

tages when compared with many other receptor affinity labels. First, the active aziridinium forms are isosteric with the known potent antagonists benzilylcholine and propylbenzilylcholine, and second the reactive moiety is a direct analogue of the onium headgroup of the reversible ligands. Because of this, it is expected that the headgroup of the irreversible and reversible ligands should be bound identically in the initial reversible phase of binding. The reaction mechanism involves a nucleophilic attack by an amino acid sidechain of the receptor sequence on the aziridinium moiety. Localization of the modified residue within the receptor sequence should therefore provide accurate positional information about the location of the onium headgroup in the binding site.

Peptide mapping and sequencing

The [³H]PrBCM-receptor bond can be broken by nucleophiles

It has been shown previously that the link formed between [³H]PrBCM and the purified mAChR from rat forebrain may be cleaved by hydroxylamine under denaturing conditions⁶. This is consistent with the alkylating agent forming an ester link with acidic residue(s) in the binding site. Other forms of linkage which might be considered possible *a priori*, particularly thioether bonds with cysteine residues, are not intrinsically vulnerable to nucleophilic attack.

Cleavage of the mAChR sequence with a lysine-specific protease

The above observation provided circumstantial evidence for modification of an acidic residue. To localize the modified residues within the sequence, mAChRs were purified from rat forebrain and striatum, labelled with [³H]PrBCM and digested with a lysine-

specific endoprotease, Lys-C, which cleaves on the C-terminal side of lysine residues. The conditions employed were such that the enzyme appeared to cleave at clusters of basic residues, incorporating one or more lysines.

Two major classes of peptide were obtained, one of high molecular weight (greater than 27 kDa) and one of low molecular weight (less than 10 kDa). Virtually all of the [³H]PrBCM label was associated with the high molecular weight peptides. In particular, two peptides of molecular weight 28 kDa and 34 kDa were identified, both of which were resistant to further digestion.

These peptides retained the carbohydrate residues which are characteristic of the mAChRs, and must therefore have extended to the N-terminus of the sequence. After deglycosylation, the molecular weights of the peptides were estimated to be 22 kDa and 14 kDa respectively, indicating the presence of ca. 200 and 130 amino acid residues, respectively. It was proposed that the peptides originate from different co-purified mAChR subtypes in the rat forebrain preparation, the longer one being generated by cleavage at Lys236 in the m4 sequence, and the shorter by cleavage at Lys136 in the m1 sequence (Fig. 1). mRNAs for both subtypes are expressed at comparatively high levels in rat forebrain². By both pharmacological and functional criteria, it seems safe to assume that m1 and m4 mAChR proteins are present in forebrain membranes, and are likely to be present in the purified preparation.

Subcleavage and sequencing

To obtain sequenceable peptides, the 34 kDa and 28 kDa peptides were partially separated from one another by high-resolution gel filtration and subcleaved with cyanogen bromide, an agent which, under the conditions used, cleaves specifically on the
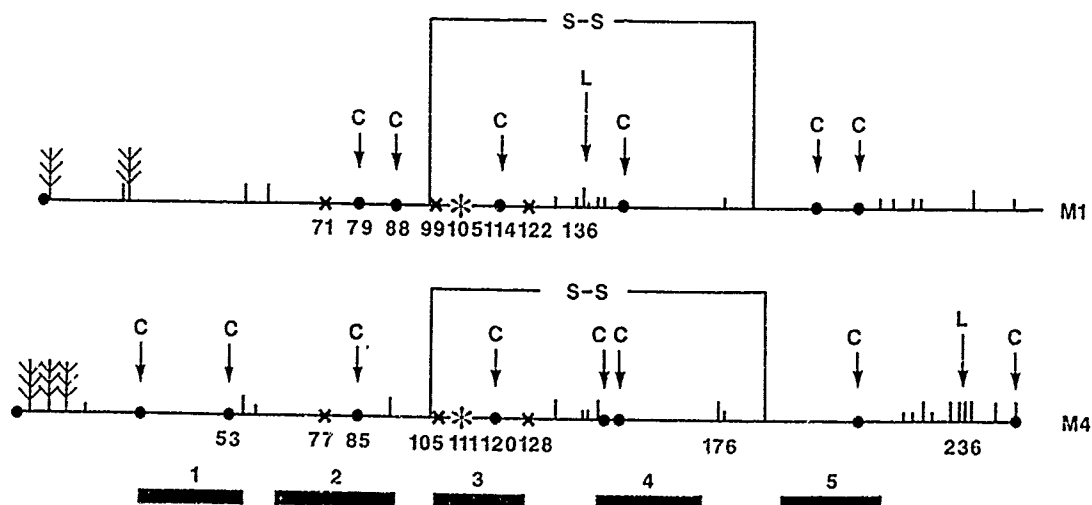
Fig 1 Location of important features within the first 250 residues of the rat m1 and m4 mAChR sequences. The probable Lys-C cleavage sites are marked (L). Note that these occur where there are clusters of basic residues as at Lys 136 (m1) or Lys 236 (m4). Potential CNBr cleavage sites are marked (C). Aspartic acid residues are indicated by ✗, and where they are the major site of alkylation by [³H]PrBCM they are indicated by ✳. N-glycosylation sites, a putative disulfide bond, and the position of the transmembrane helices are also shown

TABLE I Predicted and actual sequencing release patterns

mAChR subtype	Site of cleavage	Site of [³H]-PrBCM labelling	Sequencing predicted cycle no.	Release found cycle no.
m1	Met88	Asp99	11	11
m1	Met88	Asp105	17	17
m1	Met79	Asp99	20	
m4	Met85	Asp105	20	20
m1	Met79	Asp105	26	
m4	Met85	Asp111	26	26

C-terminal side of methionine residues. CNBr cleavage sites are marked in Fig. 1.

The fractions enriched in the 28 kDa peptide yielded primarily a [³H]PrBCM-labelled CNBr cleavage product of 2.7 kDa molecular weight, corresponding to ca. 25 amino acids. From fractions enriched in the 34 kDa peptide, an additional [³H]PrBCM-labelled CNBr cleavage product of 3.9 kDa, or ca. 35 amino acids was obtained. The probable origin of these peptides is by cleavage at Met88 and Met114 in the m1 sequence, and at Met85 and Met120 in the m4 sequence, respectively. Both of these sequences contain two Asp residues, namely Asp99 and Asp105 (m1 sequence) or Asp105 and Asp111 (m4 sequence), both of which are candidates for [³H]PrBCM alkylation.

To identify the labelling site, the cleavage products were subjected to pulsed liquid-phase sequencing. From CNBr cleavages of fractions enriched in the 34 kDa peptide, there was clear, unequivocal Edman degradation-specific release of ³H after 26 sequencing cycles (Hulme, E.C., Curtis, C.A.M., Wheatley, M.,

Harris, A.C.M. and Aitken, A., unpublished). From cleavages of fractions enriched in the 28 kDa peptide, there was also release after 26 cycles, but also statistically-significant release after 11, 17 and 20 cycles in some experiments. However, in general, these subsidiary sites of release have proved less reproducible than release after 26 cycles of Edman degradation. These results are summarized in Table I.

Release after 26 cycles supports [³H]PrBCM labelling of Asp105 (m1) or Asp111 (m4) with cleavage at the conserved Met residue 71 (m1) or 77 (m4). It should be noted that cleavage of the m1 sequence at the non-conserved residue Met88 proceeds less readily than cleavage at Met79, particularly in the larger-scale cleavages necessary for sequencing work. This conserved Asp residue thus seems to be the primary site of labelling. However, the observation of release after 11 or 20 sequencing cycles is compatible with the presence of a proportion of the label at the second Asp residue (Asp99, m1 sequence). So far, there has only been evidence for this in fractions derived from cleavage of the 28 kDa precursor peptide, which is believed to originate from the m1 sequence.

Structural implications

The primary site of [³H]PrBCM labelling, namely Asp105 (m1)/Asp111 (m4) is located within transmembrane helix 3 of the mAChR sequences (Fig. 1). The presence of an acidic residue at this position is characteristic of the cationic amine receptor sequences[1,7]. Mutation of the corresponding residue in the β-adrenergic receptor to Asn greatly reduced the affinity of the mutant receptor for both agonists and antagonists[1]. The evidence thus strongly supports a role for this Asp residue in binding the headgroup of muscarinic ligands and other cationic amines.
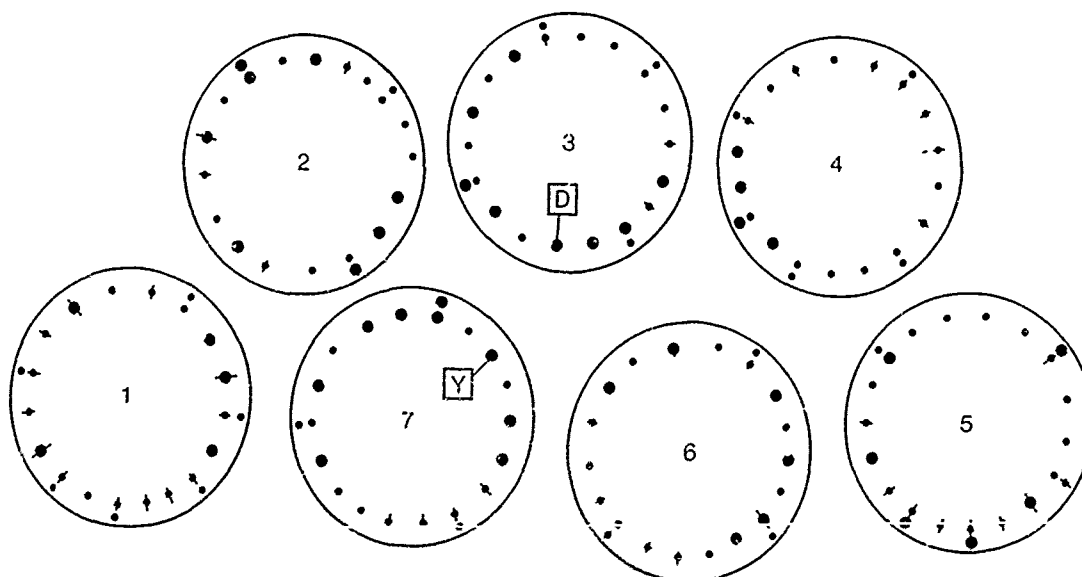

Fig 2 A helical wheel model of the m1 mAChR ● Potential hydrogen bonding residues ● Non-polar residues A line through the symbol indicates non-conservation in the m1–m5 sequences.

The tentative evidence for labelling of the more superficially-located Asp residue in the m1 sequence is harder to interpret. There is no reason to suppose that this residue plays a major part in the equilibrium binding of ligands. However, because of its proximity to the binding site Asp, and its location at the mouth of the binding site, it may have a secondary role, possibly participating in the transition state of ligand binding.

It is interesting to note that there is a second amino acid residue which appears to be characteristic of cationic amine receptors[7]. This is Tyr408 (m1 sequence) This residue corresponds to the retinal attachment lysine in the rhodopsin family of proteins[8]. It always occurs in conjunction with the transmembrane 3 binding site Asp residue, but is not found in G protein-coupled receptors which are not cationic amine receptors[7]. The co-conservation of the transmembrane 3 Asp and the transmembrane 7 Tyr may indicate a structural association, e.g. a hydrogen bond and justify between them. The existence of such a structurally-important hydrogen bond would have major implications for the interpretation of site-directed mutagenesis studies on this class of receptors.

A helical wheel model of the mAChRs

The transmembrane regions of the mAChRs vary in their hydrophobicity. Transmembrane 7 contains a particularly high proportion of hydrophilic amino acids. Plotting the sequences of the transmembrane regions on helical nets shows that transmembrane 3 and transmembrane 7 are both strongly amphiphilic, with a face consisting of hydrogen bonding residues (Tyr, Ser, Thr, Asn, Trp, Asp) running the entire length of the helix. The remainder of the helix surface is uniformly hydrophobic Hydrophilic patches can be identified on other helices. e.g. transmembrane 6 and transmembrane 1. In transmembrane 6, a cluster of hydrophilic residues around the proline induces a marked fluctuation in the hydropathicity plot.

If the transmembrane sequences of the mAChRs are drawn on helical wheel projections, it is possible to arrange them so that most of the conserved residues are on the inside of the structure (or at helix boundaries), whilst most of the non-conserved residues are on the outside, facing the lipid bilayer (Fig 2) It is then found that the majority (ca. 30 out of 47) of the potential hydrogen-bonding side chains face one another around a central pore-like cavity. Several of the apparently outward-facing hydrogen-bonding residues are at or near helix boundaries, where they may be able to interact with the aqueous phase.

The transmembrane 7 Tyr and the transmembrane 3 Asp can be bought into close proximity, in accord with the hypothesis that the two residues may interact in the cationic amine receptor sequences. There are scarcely any non-conserved residues in the central pore-like region. If this model is valid the mAChRs enclose a highly-conserved, hydrophilic cavity, which seems to be involved in ligand binding. Most of the variation in the transmembrane regions occurs in hydrophobic portions of the structure facing the lipid bilayer, where they are not subject to the necessities of interhelix hydrogen bonding, or packing constraints. In many respects, this form of protein organization is the obverse of that of soluble globular proteins, in which the hydrophilic residues tend to be on the surface, whilst the hydrophobic amino acids are close-packed together in the core.

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Genetic approaches to the determination of structure–function relationships of G protein-coupled receptors

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The β-adrenergic receptor (βAR), which has been extensively characterized pharmacologically, serves as a useful model system for the analysis of the structure–function relationships of G protein-coupled receptors. Genetic and biochemical analysis has revealed that the ligand binding domain of the receptor involves residues within the hydrophobic transmembrane core of the protein. Molecular substitution experiments suggest that adrenergic agonists and antagonists are anchored to the receptor through an ionic interaction between Asp113 in the third hydrophobic region of the receptor and the protonated amine group of the ligand. In addition, catecholamine agonists are bound through hydrogen bonding interactions between two serine residues in the fifth hydrophobic domain of the receptor and the catechol hydroxyl groups of the ligand. Agonist-mediated activation of the G protein G_s requires residues within the cytoplasmic loop linking the fifth and sixth transmembrane helices which are predicted to form amphipathic α-helices. The strong structural similarities among G protein-coupled receptors imply that the information gained from genetic analysis of the βAR should be applicable to other hormone and neurotransmitter receptors of this class.

Many hormones and neurotransmitters, both peptides and small molecules, mediate their intracellular effects through interactions with guanine nucleotide binding regulatory protein (G-protein) linked receptors[1]. The binding of hormone agonists to cell surface receptors leads to the formation of a high-affinity complex between the ligand-bound receptor and one of a family of related G proteins. The formation of this ternary hormone–receptor–G protein complex results in the release of GDP from the guanine nucleotide binding site of the G protein, allowing GTP to bind. The GTP-bound form of the G protein is the activated form, which dissociates from the receptor and interacts with specific effector enzymes, modulating the levels of intracellular second messengers. At least 50 different G protein-coupled receptor systems have been identified to date. In addition, at least 10 different G proteins link cell surface receptors to a variety of effector pathways, including adenylyl and guanylyl cyclase, K$^+$ and Ca^{2+} channels, phosphodiesterases, and phospholipases A and C. Thus, the signal transduction pathway involving G protein-coupled receptors represents one of the major mechanisms of transmembrane signaling in cellular systems.

Receptor structure

The recent cloning of the genes encoding several of these receptors, G proteins, and effector enzymes has revealed structural similarities within each of these classes of proteins, presumably reflecting their common mechanisms of action (for review, see Ref. 2). All of the G protein-coupled receptors whose primary sequences have been determined by this method are characterized by the appearance of 7 stretches of 20–25 hydrophobic amino acid residues which would be predicted to form transmembrane α-helices. Most of the sequence conservation among these receptors resides within these putative 7 transmembrane domains, which are separated by 8 more divergent hydrophilic regions, predicted to form alternating extracellular and intracellular loops. A model for the transmembrane orientation of the β-adrenergic receptor (βAR) is shown in Fig. 1. This model for the structure of the βAR is based on analogies with structural data for bacteriorhodopsin, which has a similar hydropathy profile[3]. Similar transmembrane topographies have been predicted for all of the G protein-linked receptors whose sequences are currently known. The extramembraneous exposure of many, but not all, of the hydrophilic loops of the receptor has been confirmed by biochemical or immunological techniques. In addition, the N-terminal hydrophilic domains of these receptors contain consensus glycosylation sites, which have been demonstrated to be glycosylated in the βAR[4] and rhodopsin[5], suggesting that the N-termini of these proteins are exposed extracellularly. The predicted transmembrane topography would then dictate an alternating cytoplasmic and extracellular exposure for the remaining hydrophilic loops, leaving the cytoplasmic tail of the receptor exposed intracellularly. It is important to emphasize that the structure of the receptor which is presented in Fig. 1 depicts a working model for the βAR, with the actual secondary and tertiary structure of the protein remaining to be determined. However, as detailed below, such a model has proven useful in transferring information obtained for one G protein-linked receptor to other related systems.

Ligand binding domain

Because of the wealth of structure–activity information available for β-adrenergic ligands, the βAR, which

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couples to the G protein G_s to activate adenylyl cyclase, represents a useful model system for the determination of structure–function relationships of G protein-coupled receptors. In order to determine which regions of the receptor protein are required for ligand binding to occur, deletions were made in the βAR gene to remove each of the hydrophilic and hydrophobic domains of the protein independently. The resulting mutant receptors were expressed in mammalian cells and their biochemical and pharmacological properties determined[6]. The ability of the mutant receptors to be fully glycosylated and inserted into the cell membrane was assessed by the appearance of the protein as a broad band with an apparent molecular weight of 67 kDa on SDS–polyacrylamide gels, detected by protein immunoblotting using an antibody specific for the βAR[6]. In contrast, nonglycosylated receptor migrated as a sharp immunoreactive band at 47 kDa. Failure of a mutant receptor to be normally glycosylated or membrane associated was taken as diagnostic of an adverse affect of the mutation on the tertiary structure of the receptor. By these criteria, deletions of any of the 7 hydrophobic regions of the βAR, either alone or in pairs, or of regions which would be predicted to form junctions between the membrane-spanning helices and the extramembraneous loops, resulted in a failure of the protein to fold properly and be inserted into the membrane[6,7]. These data suggested that the hydrophobic putative transmembrane domains are critical for the structural integrity of the receptor protein. On the other hand, most of the hydrophilic regions of the receptor could be deleted without adversely affecting either the appearance of the βAR in the membrane or the ligand binding properties of the receptor. These experiments demonstrated that most of the extramembraneous hydrophilic regions of the protein are not required for ligand binding to occur, indicating that the ligand binding domain of the receptor is located within its hydrophobic transmembrane core[6,7]. This result is of interest in light of data indicating that the binding site of retinal in rhodopsin, which couples to the G protein transducin, is also located within the hydrophobic core of the protein[8]. Retinal interacts with opsin through the formation of a Schiff base with the side chain of Lys296 in the seventh hydrophobic domain. Furthermore, biophysical studies suggest that the bound chromophore in rhodopsin is buried approximately 30% into the membrane bilayer. The localization of the binding sites of both the βAR and rhodopsin to the hydrophobic regions of these proteins supports the hypothesis that the similar structural features of these two proteins reflect similarities in function and suggests the possibility of extrapolating structural data from one G protein-coupled receptor system to another.

In order to define the specific molecular interactions involved in anchoring the ligand to its binding site in the receptor, a series of site-specific mutations was introduced into the βAR gene, resulting in substitutions of single amino acid residues within the receptor. The sites of the mutations were selected by analysis of pharmacophore maps of β-adrenergic ligands, as outlined in Fig. 2. The endogenous agonists for the βAR are catecholamines, characterized by a protonated amine group which is separated from a catechol ring by

a β-hydroxyethyl chain[9]. The identity of the substituent on the amine distinguishes the endogenous ligands epinephrine and norepinephrine, defining the two subtypes of the βAR [β₁AR: K_d(epinephrine) = K_d(norepinephrine); β₂AR: K_d(epinephrine) < K_d(norepinephrine)]. The protonated amine and the β-hydroxyl group of the ligand are important for both agonist and antagonist binding to the receptor, whereas the catechol moiety is characteristic of agonists. Furthermore, antagonists are characterized by an increased hydrophobicity of the aromatic ring structure and an increased distance between the aromatic ring and the amine group. This is usually achieved by the substitution of a phenoxymethylene moiety for the phenyl ring of the agonists. Thus, specific molecular requirements for ligand binding to the βAR include an interaction with the protonated amine, hydrogen bonding interactions with the β-hydroxyl and catechol hydroxyl groups, and agonist- or antagonist-specific aromatic interactions with the ring systems (Fig. 2).

Because some of the structural features of adrenergic ligands are shared by ligands which recognize other G protein-coupled receptors, one might expect that amino acid residues in the receptor which interact with certain functional groups on the ligands would be selectively conserved with other G protein-coupled receptors which bind similar ligands. By this reasoning, the amino acid residue(s) in the βAR which interact with the protonated amine group of the ligand would be predicted to be conserved with other G protein-linked receptors which bind protonated amine ligands,
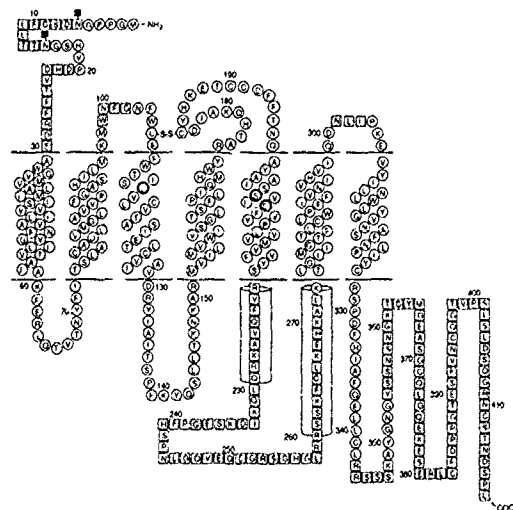
Fig. 1 Model for the transmembrane topology of the βAR The horizontal lines represent the limits of the membrane bilayer, with the extracellular space at the top of the page Amino acid residues represented by squares could be deleted from the receptor without affecting ligand binding or protein folding. Asp113, Ser204, and Ser207 are encircled in bold. The cylinders outline the regions in the third intracellular loop which are required for G protein coupling Reprinted with permission of the American Journal of Respiratory Cell and Molecular Biology, 1989; Vol 1, No. 2

such as the α-adrenergic (αAR), muscarinic acetylcholine (MAChR), 5-HT, and dopamine receptors, but not with the receptors whose ligands are not protonated amines. Similarly, the residue(s) involved in binding to the β-hydroxyl group would be expected to be specific for the αAR and βAR, whereas, residue(s) which interact with the catechol ring of adrenergic agonists might be conserved among the αAR, βAR, and the dopamine receptor. The structure–function analysis of the βAR in our laboratory has focused on the identification of specific amino acid residues in the receptor responsible for these molecular interactions with the ligand.

In order to identify amino acid residues involved in anchoring the protonated amine group of the ligand to the receptor, mutations were introduced into the βAR gene to replace acidic amino acids located within the hydrophobic domain of the receptor with neutral residues[10,11]. Three such acidic residues, Asp79, Glu107, and Asp113 in the hamster βAR, are conserved among adrenergic receptors, making them candidate counter-ions for the protonated amine moiety of the ligand. Mutations were introduced at these positions, producing [Ala^{79}]βAR, [Ala^{107}]βAR and [Asn^{113}]βAR, which were expressed in mammalian cells[10]. Examination of the ligand binding properties of these mutant receptors revealed an absolute requirement for Asp113 in the third putative transmembrane domain of the receptor in order for normal ligand binding to occur. Substitution of Asp113 with an Asn residue resulted in a 10 000-fold decrease in the affinity of the receptor for adrenergic agonists[11]. Substitution of a Glu residue at this position caused a 100–300-fold decrease in the affinity of activation by adrenergic agonists, suggesting that both the negative charge at position 113 and the positioning of that charge within the receptor structure are important in determining the affinity of the receptor for the ligand. The affinity of the receptor for adrenergic antagonists was also decreased dramatically by the

Fig. 2 Map of ligand–receptor interactions in the binding site of the βAR. A catecholamine ligand is shown in a hypothetical binding site intercalated among the transmembrane helices of the receptor. Each of the large semicircles represents a transmembrane helix of the receptor, inscribed with the type of binding interaction expected. Other G protein-coupled receptors which would be expected to have similar interactions with their specific ligands are designated in boxes next to each helix.

substitution of a Glu or an Asn residue for Asp113, indicating that this residue is involved in the binding of both agonists and antagonists to the βAR.

Interestingly, several adrenergic antagonists, such as alprenolol and pindolol, displayed partial agonist activity at [Glu^{113}]βAR, stimulating adenylyl cyclase activation by this mutant receptor to levels of 10–35% maximal[12]. These ligands were full antagonists at the wild-type βAR and at [Asn^{113}]βAR. Thus, the substitution of a Glu for Asp113, altering the distance between the negative charge and the protein backbone, allowed the βAR to assume its active conformation upon binding antagonist ligands which normally do not activate the wild-type receptor. These results argue for the existence of overlapping binding sites for adrenergic agonists and antagonists and suggest that the position of the negative charge at position 113 is important in determining the orientation of the ligand in the binding pocket of the receptor. In contrast to the absolute requirement for the acidic residue at position 113, substitution of Asp79 with Ala reduced the affinity of the receptor for agonists by tenfold without affecting antagonist binding, and substitution of Glu107 with Ala had no effect on ligand binding to the βAR[10]. Thus, it appears that adrenergic agonists and antagonists interact with the receptor at position 113, probably through the formation of an ion pair between the carboxylate side of Asp113 and the protonated amine group of the ligand. This assignment is supported by the observation that an Asp residue at the position analogous to that of Asp113 in the βAR is present in all known G protein-coupled receptors which bind protonated amine ligands, including the αAR, βAR, MAChR, dopamine receptor, and 5-HT receptor, but is not present in those receptors whose ligands are not protonated amines, such as the opsins, substance K, and angiotensin receptors[11].

A similar strategy of combined genetic and biochemical analysis has led to the identification of two Ser residues in the fifth hydrophobic domain of the βAR which are required for the normal binding and activation by catecholamine agonists[13]. Substitution of either Ser204 or Ser207 with Ala residues decreased the affinity and efficacy of agonists at the receptor, without affecting antagonist binding properties. The magnitude of the decrease in binding activity of these mutant receptors was consistent with the removal of a hydrogen bond by these mutations, suggesting that these Ser residues may be involved in stabilizing one or more of the hydroxyl moieties of the ligand in the binding pocket of the receptor (Fig. 2). The decreases in agonist affinity for these mutant receptors were additive with the decreases in affinity observed upon removal of the β-hydroxyl group from the ligands. Coupled with the absence of an effect of these mutations on antagonist binding, this observation argues that these Ser residues are not involved in interactions with the β-hydroxyl moiety. The effects of replacements of Ser204 and Ser207 on agonist affinity and efficacy were selectively mimicked by the removal of the *meta* or the *para* hydroxyl group, respectively, from the catechol ring of the ligand[13]. These data are consistent with the involvement of the hydroxyl side chains of these Ser residues in hydrogen bonding
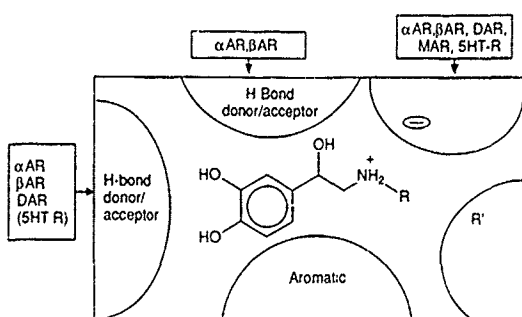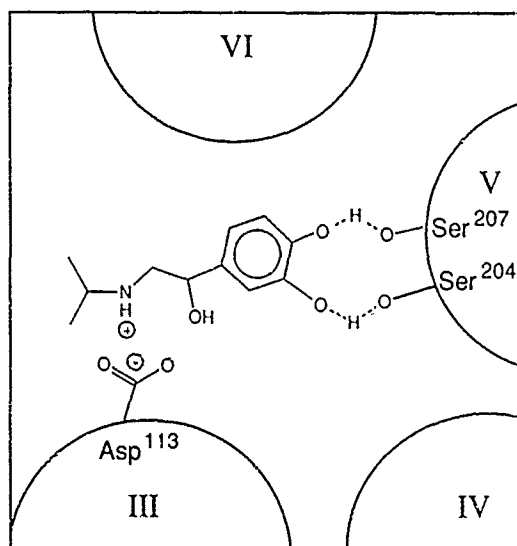
Fig 3 Model for the ligand binding site of the βAR The view of the receptor is from the extracellular face of the plasma membrane, with the positioning of the helices according to the model in Ref 3 Transmembrane helices III–VI are shown, with the locations of residues postulated to interact with the ligand drawn in the figure Reprinted with permission of the American Journal of Respiratory Cell and Molecular Biology, 1989, Vol 1, No. 2

interactions with the catechol hydroxyl groups of the catecholamine ligand. One hydrogen bond would be formed between the side chain of Ser204 and the *meta* hydroxyl group of the ligand, with a second hydrogen bond linking the *para* hydroxyl group of the ligand to the hydroxyl side chain of Ser207. Further support for these assignments arises from the observation that Ser residues analogous to Ser204 and Ser207 are present in the fifth transmembrane helices of all known G protein-coupled receptors wh ch bind catecholamine ligands, including the αAR, βAR, and dopamine receptor, but are absent from those receptors which do not interact with catechol rings, such as the MAChR, opsins, and substance K receptor[13]

Taken together, these data support the model for the ligand binding site of the βAR which is shown in Fig. 3 (Ref. 2). The ligand binding pocket resides within the hydrophobic core of the protein, with contributions from amino acids located on various transmembrane helices. According to this model, the protonated amine group of the ligand forms an ion pair with the carboxylate side chain of Asp113 in transmembrane helix 3, whereas the catechol ring is anchored by hydrogen bonding interactions between the catechol hydroxyl groups and the hydroxyl side chains of Ser204 and Ser207 in transmembrane helix 5. Other molecular interactions, yet to be defined, are postulated for attaching the other functional groups of the ligand within the binding pocket of the receptor. The model shown in Fig. 3 represents a functional, rather than a

rigorous structural, model for receptor–ligand interactions. The sites of interaction which are shown in the model would impose conformational constraints on the orientation of the ligand in the binding site of the receptor. Structural analysis of the ligand-bound form of the βAR will be necessary to identify these conformational constraints and to refine further our understanding of the structure of the ligand binding site of the receptor.

Molecular replacement analysis has been less successful in identifying specific interactions of the receptor with the substituent on the amine group of the ligand. As mentioned above, this substituent provides the molecular basis for the selectivity of the β₁ and β₂ receptor subtypes for the endogenous agonists epinephrine, which has a methyl group at this position, and norepinephrine, which has a hydrogen atom attached to the amine. Pharmacological analysis of chimeric receptors having increasingly long N-terminal portions of the β₂ receptor attached to the complementary C-terminal portions of the β₁AR demonstrated that, as helix 4 of the receptor was changed from β₁ to β₂, the affinities of the agonists for the receptor became more characteristic of the β₂AR[14]. Subsequent molecular replacements of individual helices demonstrated that this alteration in subtype specificity could be achieved by substituting helices 4 and 5 of the β₂AR with the analogous regions of the β₁AR[15]. In contrast, smaller helical replacements or single amino acid substitutions in the receptor failed to affect subtype specificity[15]. These data indicate that the subtype-specific differences in catecholamine agonist binding cannot be attributed to a single molecular interaction between the ligand and any amino acid residue which is divergent between the β₁ and β₂ receptors. Rather, this recognition site appears to arise from a conformation of the receptor which is influenced by sequences within transmembrane helices 4 and 5[15]

Interactions with G proteins

The binding of agonists to the βAR promotes its interaction with G_s, leading to the activation of adenylyl cyclase. Antagonists do not promote this functional interaction with the G protein. Deletion mutagenesis and molecular replacements have been used to identify the site(s) of interaction between the receptor and the G protein. Such studies have indicated that most of the putative extracellular hydrophilic domains of the βAR can be deleted without affecting G protein coupling. A large deletion within the region predicted to form the third intracellular loop of the receptor, however, results in a complete loss of G protein coupling and adenylyl cyclase stimulation by the mutant receptor[16] This result is in agreement with experiments showing that proteolytic cleavage within the analogous third intracellular loop of rhodopsin disrupts its ability to couple to transducin[8]. Smaller deletions of 10–12 amino acids within this internal loop of the βAR revealed that the 8 residues at the N-terminus of this loop cannot be deleted without abolishing the coupling of the receptor to the G_s-adenylyl cyclase system[17]. In addition, a deletion of 12 amino acid residues at the C-terminus of this loop significantly attenuates the coupling of the receptor to

G_s. In contrast, deletions within the central portion of the third intracellular loop do not affect the coupling of the βAR to G_s. Therefore, the regions at the N- and C-terminal portions of the third intracellular loop of the receptor appear to be required for normal coupling to G proteins[17]. Most of the C-terminal tail of the βAR was determined to be dispensable for G protein coupling, although the region of the C-terminus immediately adjacent to the seventh transmembrane domain was critical for protein processing and could not be analyzed by this technique[16]. Replacement of the N-terminal region of the third intracellular loop of the βAR with the analogous sequence of the m1 muscarinic receptor, which couples to G_p to stimulate phospholipase C, failed to restore G protein coupling to the mutant receptor[18]. In contrast, molecular replacements of the C-terminal region or of the second intracellular loop of the βAR with the analogous regions of the m1 muscarinic receptor resulted in hybrid receptors which bound adrenergic ligands and coupled normally to adenylyl cyclase activation, suggesting that these regions are not responsible for directing the specificity of G protein coupling. Consistent with these results, molecular replacement studies on hybrid m1–m2 muscarinic receptor revealed that the specificity for G protein recognition correlated with the source of the third intracellular loop[19]. Genetic analysis utilizing single amino acid substitutions in this region will be necessary to define further the molecular basis for G protein specificity.

The regions at the N- and C-termini of the third intracellular loop of the βAR which are implicated in the coupling of the receptor to G_s would be predicted by secondary structure prediction algorithms to form amphipathic α-helices[16]. This structural feature is of interest in light of the observation that the amphipathic peptide mastoparan is able to activate the G protein, G_o[20]. Although this hydrophilic loop represents one of the most divergent regions of the primary sequence of G protein-coupled receptors, all of these receptors would be predicted to have amphipathic helices at this position[2]. These observations suggest that the mechanism of coupling between receptors and G proteins may involve interactions with these amphipathic domains. According to the structural model proposed for the βAR, these amphipathic regions would be predicted to form cytoplasmic α-helical extensions of the fifth and sixth transmembrane helices of the receptor (Fig. 1). This location of a region involved in G protein coupling adjacent to the fifth hydrophobic domain of the βAR suggests a mechanism by which agonist binding to the receptor might stimulate G protein coupling[13]. The interaction of the ligand with the serine residues in transmembrane helix 5 could cause a conformational change in this helix, which could be transmitted to the

amphipathic region at the bottom of helix 5, allowing the receptor to interact with G_s. Antagonists, which do not interact with the Ser residues in transmembrane helix 5, would not be expected to cause this conformational change and, hence, would not promote G protein activation.

The nature of the conformational changes which accompany agonist binding to the receptor, and detailed investigation of this hypothesis for receptor activation, will await biophysical analysis of both the receptor and the G protein. The demonstrated significance of the third intracellular loop in the coupling of several different receptors to G proteins, and the involvement of Ser residues in the fifth hydrophobic domain of the βAR in agonist binding and activation, predicts that similar agonist-specific interactions involving residues in the fifth transmembrane domains of other G protein-coupled receptors will exist. The identification of these and other binding interactions should provide insights into the molecular basis for the ligand recognition and G protein activation functions of these receptors and lead to the development of new classes of therapeutic agents which act to potentiate or to disrupt these transmembrane signaling processes.

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The modes of binding of ligands to cardiac muscarinic receptors

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Ionizable groups on the cardiac M_2 muscarinic receptor which regulate the binding of ligands have been examined by studying the pH dependence of the ligand affinity constants. The presence of three titratable residues (approximate pK values, 5.4, 6.8 and 7.5) whose protonation modulates antagonist binding has been demonstrated. Cardioselective antagonists are selectively affected by the protonation state of the pK 6.8 residue, whereas the binding of antagonists having differing selectivities is more strongly affected by protonation of the pK 5.4 residue on cardiac receptors. Methoctramine is capable of binding to both the pK 5.4 and 6.8 residues simultaneously. Protonation of the residue of highest pK produces a conformational change at the receptor which can affect both agonist and antagonist binding. It is now possible to demonstrate differences both in the way ligands bind to a given receptor subtype and in the way a given ligand binds to different subtypes.

For many years the dogma has been that a large contribution of the binding energy of muscarinic ligands to their receptor comes from the interaction of a positively charged group with a negatively charged amino acid residue on the receptor[1]. The sequence determination of five molecular species of muscarinic receptors (for reviews see Refs 2 and 3) and the ability to mutagenize these receptors now allows the possibility of identifying the negatively charged residue or residues in order to begin to build a picture of the binding site and discover differences in the structures of the subtypes which may be exploited in the binding of selective ligands.

The problem has been approached by using the aziridinium ion of [³H]propylbenzilylcholine mustard to label covalently specific residues on purified cortical muscarinic receptors[4,5]. This paper presents the preliminary results of a complementary study in which the effects of the protonation state of the receptor on the binding of muscarinic ligands has been examined.

General approach

It was decided to investigate initially the binding of ligands to rat cardiac muscarinic receptors. The binding properties of these receptors have been intensively characterized (see for example Refs 7–11) and reflect a population of predominantly M_2 receptors. In subsequent studies we have examined the M_1 muscarinic receptors in rat cerebral cortex, the receptors in rat lacrimal glands (mainly M_3) and muscarinic receptors on CHO cells which have been stably transfected with muscarinic receptor genes.

The buffer system used was a borate/citrate/phosphate system[12] which has a constant ionic strength over the pH range 3–11, thereby minimizing the variation of affinity constants with ionic strength[13].

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The ligands chosen to investigate exhibit a range of selectivities for M_1, M_2 and M_3 receptors as well as varying in the nature of the nitrogen moiety (quaternary, tertiary or secondary) in their structures

The binding of competitive antagonists to M_2 receptors

The radioligand used in most of our binding assays was [³H]N–methylscopolamine ([³H]NMS). Receptor specific binding to M_2 receptors (i.e. that component of binding of [³H]NMS which was inhibited by 10^{-6} M 3-quinuclidinylbenzilate, QNB) was detectable over the pH range 4.5–9. The binding was relatively stable at 30°C in the time range of 10 min to 1 h but a loss of specific binding was observed at the longer incubation times and lower pHs. Saturation curves for [³H]NMS as a function of pH were constructed. Above pH 5 the estimated number of binding sites was independent of pH. The affinity constant (K_a) of [³H]NMS was independent of pH above pH 7 but decreased at lower pHs to such an extent that it could no longer be measured accurately below pH 4.5 (Fig. 1).

The gradient of the log–log plot in Figure 1 approaches unity at lower pHs This finding is compatible with one residue on the receptor being protonated in the pH range 5–7 and inhibiting the binding of [³H]NMS. Indeed, the data in Figure 1 are fitted well by a theoretical curve in which the ionizable group on the receptor has a pK of 5.9. Thus a proton is behaving as a competitive antagonist! In studies on the brain receptors where the receptor density, stability and the [³H]NMS affinity constant are all greater than for the heart receptors, it has been possible to demonstrate the apparent competitive action of protons down to pH 3 The changes in [³H]NMS affinity constant with pH are also reversible

When the binding of pirenzepine was examined in competition experiments with [³H]NMS, the log K_a–pH curve was bell-shaped (Fig. 2). The form of this curve resulted from the deprotonation of pirenzepine at high pH and the protonation of the receptor at low pH both
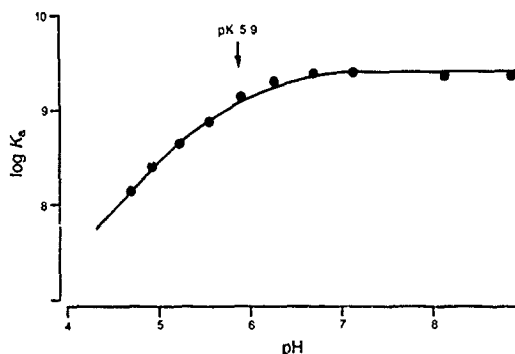
Fig 1 The pH dependence of the affinity constant (K_a) for the binding of [³H]N–methylscopolamine to rat cardiac muscarinic receptors.

resulting in decreased binding of pirenzepine. The data are compatible with a model in which pirenzepine has a pK of 8.2–8.3 and the receptor has a pK of 6.1. The former value is in agreement with pK estimates obtained by electrometric titration[14]. The pK value calculated for the receptor agrees reasonably well with that calculated using NMS as the ligand. From these limited data one might conclude (erroneously as it turns out) that a single protonable group with a pK of ~6 regulates antagonist binding to cardiac receptors.

A surprising finding emerged when the pH dependence of the binding of a number of other antagonists was examined. The apparent pK calculated for the receptor depended on the nature of the ligand. This is illustrated for AF-DX 116 and benzhexol (Fig. 3). The log K_a for AF-DX 116 shows a much greater pH dependence (50-fold) than that of benzhexol (4-fold) over the pH range 5–8. Although both these antagonists are tertiary amines, the pK values for protonation of the relevant N atoms are expected to be well above 9. Hence, over the whole pH range illustrated, they behave as positively charged ligands (cf [³H]NMS, Fig. 1).
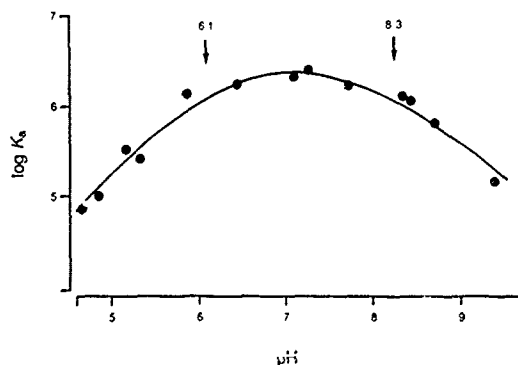
The apparent pK values for the receptor, calculated

Fig 2 The pH dependence of the affinity constant (K_a) for the binding of pirenzepine to rat cardiac muscarinic receptors. The estimated pK value of pirenzepine (8 3) and the apparent pK value of the receptor (6.1) are indicated by the arrows

from the benzhexol and AF-DX 116 are 5.6 and 6.8. This means that at least two ionizable groups on the receptor must be capable of regulating antagonist binding, and that benzhexol and AF-DX 116 bind in a sufficiently different manner to cardiac muscarinic receptors so that protonation of different amino acid residues on the receptor can differentially affect the binding of one ligand or the other. When the binding of a number of antagonists is compared it is found that they form two groups; those antagonists which give an apparent pK for the receptor of 5.5–6.1 and those grouping in the pH range 6.5–6.8. It seems that the range of apparent pK values within a group are real and not due to experimental error. Hence it is not possible to draw the simple conclusion that antagonists, depending on their structure, are capable of interacting exclusively with one or the other of two ionizable groups.

A most important feature of the division of the binding behaviour of antagonists into two groups is that only cardioselective antagonists give high apparent pK values. Conversely, no cardioselective antagonists have been found in the group of antagonists giving low apparent pK values. Despite the relatively small number of antagonists examined (15) it seems as if cardioselectivity may be associated with the interaction with the ionizing group of higher pK

The binding of gallamine to M₂ receptors

The finding of the existence and importance of two ionizing groups for the binding of antagonists suggested that our previous demonstration of two binding sites for ligands on muscarinic receptors[15] might be related. The latter finding was based initially on the ability to demonstrate the formation of ternary complexes between the receptor and two ligands, e.g. [³H]NMS and gallamine.

The interaction between gallamine and muscarinic receptors is characterized by two affinity constants, the binding of gallamine to the unliganded receptor (K_1) and to the liganded receptor (K_2). The ratio of these affinity constants (K_1/K_2) is the heterotropic cooperativity between the binding of gallamine and the other ligand to the receptor.

When the pH dependence of the binding of gallamine was examined we found that, as would be predicted for a cardioselective antagonist, its interaction with the unliganded cardiac receptor (K_1) gave a high apparent pK of 6.7. However this apparent pK changed to 6.2 when the pH dependence of gallamine binding to the receptor-[³H]NMS complex (K_2) was calculated. Therefore the binding of NMS to the cardiac receptor changes the apparent pK of the residue that regulates gallamine binding as well as decreasing the gallamine affinity constant.

The binding of methoctramine to M₂ receptors

Methoctramine is a cardioselective muscarinic antagonist whose tetramine structure is quite unusual[16,17]. Hence its interaction with muscarinic receptors might be expected to be different from those of other antagonists. The pH dependence of methoctramine binding shows this to be true. At high pH (8–9), the affinity is approximately constant and rises 4-fold as the pH decreases to 7, whereupon there is a very profound

decrease in affinity as the pH decreases further. This decrease is such that, at pH 4, methoctramine binds ~ 10000 times weaker than at pH 7. At low pH (4–5) the gradient of the log K_a–pH curve is 2, indicating that methoctramine is binding simultaneously to two ionizable groups with pKs above 5. A more detailed analysis suggests that these pK values are approximately 5 4 and 6.8, corresponding to the extreme values found for the antagonists described earlier. In addition, methoctramine binding appears to be allosterically regulated by yet another titratable residue with a pK of approximately 7.5. This pK value is not a protonation pK of methoctramine as equivalent pH studies of methoctramine binding to M_1 and M_3 receptors do not show evidence of this pK. Furthermore, polyamines separated by more than four methylene groups do not have pKs which are lowered considerably by protonation of adjacent amino groups. Hence protonation of the residue on the receptor with a pK of 7.5 results in an *increase* in methoctramine binding which must result from a conformational change of the receptor.

Discussion

Previous studies of the pH dependence of ligand binding to muscarinic receptors[18][19] have focused on the examination of the effect of protonation of the ligand on its affinity for the receptor. In our studies we have concentrated on protonation events at the receptor. These have not previously been examined in detail for any G-protein coupled receptor.

On cardiac muscarinic receptors, there appear to be two residues of pK values approximately 5.4 and 6.8, the protonation of which decrease antagonist binding. A few antagonists seem to be specifically affected by protonation of either one or the other residue but many antagonists give apparent pK values which are in between 5 4 and 6.8. There are two possible interpretations of these findings. Firstly, the positively charged nitrogen of the ligand may have the choice of whether it interacts with the pK 5.4 or pK 6.8 residue on the receptor This is formally equivalent to the ligand interacting initially with one residue on the receptor and the complex isomerizing such that the ligand now interacts with the other residue. The extent of isomerization would depend on the nature of the ligand and give an apparent pK which is a weighted average of 5.4 and 6 8. Some suggestive evidence for this mechanism comes from the complex binding kinetics of antagonists and the fact that [³H]propylbenzilylcholine mustard labels two different aspartate residues in m1 receptors[5].

An alternative explanation is that the ligand binds only to the pK 5.4 residue and this binding is allosterically inhibited by protonation of the pK 6.8 residue, the extent of the allosteric effect depending on the structure of the ligand. The dramatic effects on antagonist binding of the mutation of a buried aspartate residue in the putative third transmembrane segment of β-adrenoceptors[20,21] and m1 receptors (C. Fraser, pers commun) and the minor effects of the mutation of the other conserved acidic residues point to the importance of a single acidic residue for the binding of antagonists (or maybe the intrinsic structure of the receptor). Careful experimentation may allow a discrimination between these two models.
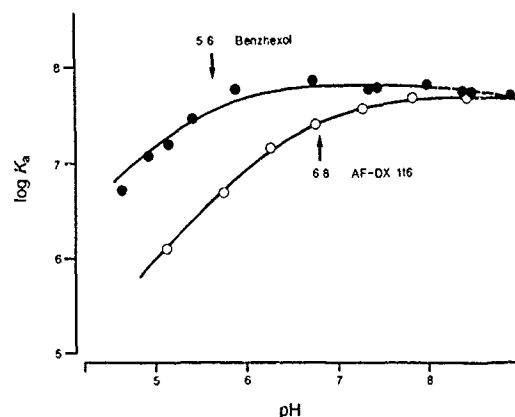

Fig 3 The pH dependence of the affinity constant for the binding of benzhexol (closed circles) and AF-DX 116 (open circles) to rat cardiac muscarinic receptors The apparent pK values of the receptor are indicated by the arrows

There is no identification as yet of the residues having the pK values of approximately 5.4 and 6.8. The two aspartate residues on the putative extracellular side of the third transmembrane segment are clear candidates on the basis of (1) their conservation in the sequences of the different muscarinic receptor subtypes and in several monoamine G-protein coupled receptors, (2) the effects of their mutagenesis to asparagine residues and (3) the protein labelling studies using [³H]propylbenzilylcholine mustard[4,5]. In this context, it is relevant to note that as early as 1974 C.R Hiley (PhD thesis, University of Cambridge, 1974) found that the alkylation of brain muscarinic receptors by [³H]propylbenzilylcholine mustard was regulated by a group with an apparent pK of 6.3. Clearly the aspartate residues would have to be in somewhat unusual environments for their pKs to be raised to such high values. Experiments in progress with mutagenized receptors may help with the identification of these residues.

The nature of the residue of even higher pK (~7.5) is also not known. Though originally detected in studies with methoctramine on M_2 receptors, we have subsequently found this residue to be important in allosterically regulating agonist binding to M_2 receptors and the binding of some other antagonists to M_1 receptors (unpublished results). Candidate residues include the other conserved aspartate residues in the second and third transmembrane segments and the apparently important conserved tyrosine residue in the seventh transmembrane segment[3]. Clearly protonation of this residue of the receptor at physiological pHs produces a conformational change in the receptor which can affect both agonist and antagonist binding.

Finally, it is of considerable interest that there appears to be a linkage between cardioselectivity of a ligand and its interaction with the pK 6.8 residue of M_2 receptors. This indicates an element of commonality in the way that cardioselective ligands bind, which is different from the way that other ligands bind to these receptors. Our recent studies on M_1 and M_3 receptors

have shown that ligands exhibit a different pK profile of binding to these receptors from that shown for M_2 receptors. It is thus possible to demonstrate differences in the way a given ligand binds to the different receptor subtypes.

This approach, which should be of general application to drug–receptor interactions, may be useful in the design of new therapeutic agents.

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Diverse functions of muscarinic acetylcholine receptor subtypes

James Lechleiter, Ernest Peralta* and David Clapham

Muscarinic acetylcholine receptor subtypes m1, m3 and m5 couple strongly to phosphatidylinositol turnover and hence to intracellular Ca^{2+} concentration via pertussis toxin (PTX) sensitive and insensitive G proteins. The m2 and m4 muscarinic receptor subtypes strongly inhibit adenylyl cyclase production via PTX sensitive G proteins. Additionally, the cardiac M_2 receptor is closely coupled to a K^+ current ($I_{K\,ACh}$). To characterize this functional diversity more completely, we measured the ACh-induced Ca^{2+} responses of cells transfected with the muscarinic receptor subtypes m1, m2, m3 and m4. As expected, cells transfected with m1 or m3 receptors exhibited large dose-dependent increases in Ca^{2+} in response to ACh application. Unexpectedly, cells transfected with m2 or m4 receptors also exhibited increases in Ca^{2+} in response to agonist application. The m2- or m4-coupled responses were smaller in amplitude, required higher concentrations of agonist and were much more sensitive to PTX treatment when compared to m1- or m3-coupled responses. We discuss this remarkable diversity of function in terms of the receptor subtype's coupling to G proteins.

Muscarinic acetylcholine receptors (mAChR) are part of a large family of receptors which transduce their cell signals through GTP-binding proteins. G protein-linked receptors comprise a class of proteins with analogous structure and sequence. Theoretically, all these receptors are made up of seven transmembrane spanning segments. Five different mAChRs have now been identified by molecular cloning. Functionally, they may be separated into two categories: m1, m3 and

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m5 receptors which couple strongly to phosphatidylinositol (PI) hydrolysis, and m2 and m4 receptors which inhibit adenylyl cyclase activity but couple poorly to PI turnover. PI turnover creates inositol trisphosphate (IP_3) which in turn leads to Ca^{2+} release from intracellular stores.

Coupling of muscarinic receptors to increases in intracellular Ca^{2+}

We have examined muscarinic receptor-induced Ca^{2+} increases in single cells using confocal microscopy and the Ca^{2+} dye FLUO-3 AM (Molecular Probes). For these experiments, human embryonic kidney (HEK)
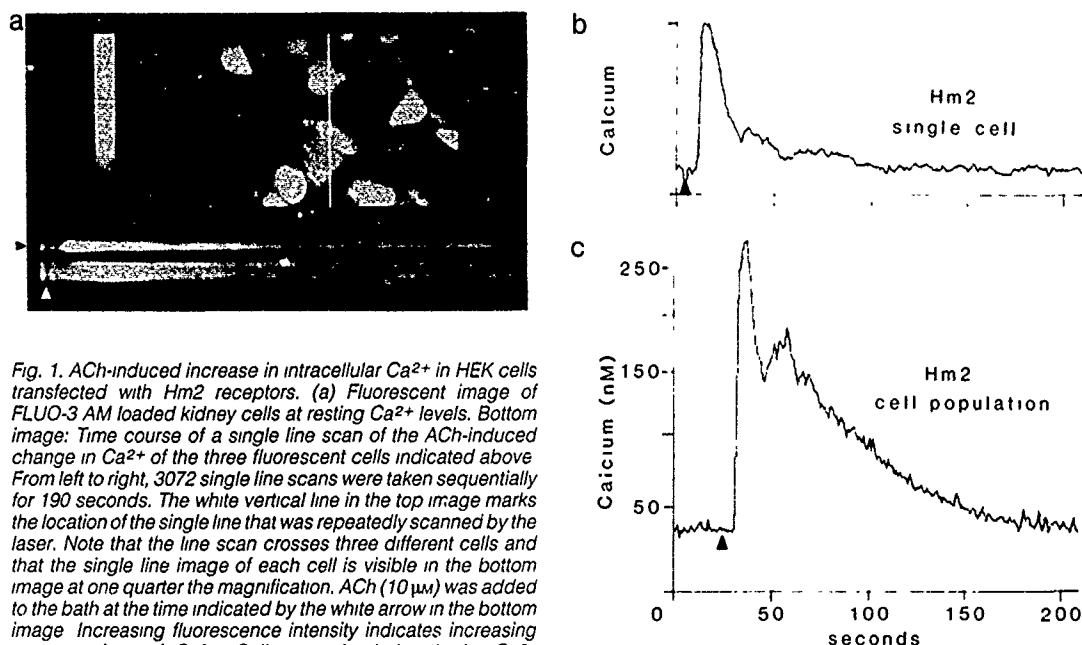
Fig. 1. ACh-induced increase in intracellular Ca^{2+} in HEK cells transfected with Hm2 receptors. (a) Fluorescent image of FLUO-3 AM loaded kidney cells at resting Ca^{2+} levels. Bottom image: Time course of a single line scan of the ACh-induced change in Ca^{2+} of the three fluorescent cells indicated above From left to right, 3072 single line scans were taken sequentially for 190 seconds. The white vertical line in the top image marks the location of the single line that was repeatedly scanned by the laser. Note that the line scan crosses three different cells and that the single line image of each cell is visible in the bottom image at one quarter the magnification. ACh (10 μM) was added to the bath at the time indicated by the white arrow in the bottom image Increasing fluorescence intensity indicates increasing concentrations of Ca^{2+}. Cells were loaded with the Ca^{2+} indicator dye FLUO-3 AM (5 μM) in the presence of Pluronic F-127 surfactant (5 μl/ml) for 1 hour at 37°C, 95% O_2, 5% CO_2 using Dulbecco's modified essential medium (DMEM) Experiments were performed at room temperature in 120 mM NaCl, < 5 mM KCl 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 r. M HEPES at pH 7 4. Cells were excited at 488 nm and fluorescence detected with standard FITC filters All images are averages of 5 frames collected on the MRC Biorad Confocal Microscope. (b) Fluorescence intensity along the top cell indicated by the black arrow in (a). The intensity profile for this single cell parallels the Ca^{2+} response observed for the cell population shown in (c) (c) ACh-induced increase in intracellular Ca^{2+} in HEK cells transfected with m2 receptors (125 000/cell) as measured by

flow cytometry. The average cellular fluorescence ratio for each second was plotted with a flow rate of 700 cells per second The fluorescence intensity ratio of the Ca^{2+} dye indicator INDO-1 AM was used to monitor intracellular Ca^{2+} ACh (10 μM) was added to the cells at 20 seconds as indicated by the black arrow Cells were loaded with INDO-1 AM (5 μM) as described for FLUO-3 AM except that for the first 30 minutes of incubation, the pH was lowered to 7.0. The Ca^{2+} dye solution was then diluted 1 1 with DMEM at pH 7 4. Cells were excited at 351 nm and fluorescence detected at 390 and 500 nm All measurements were taken with a Becton Dickinson Facstar flow cytometer

cells were stably transfected with human (H) muscarinic receptors. As observed with other PI-linked receptors, cells transfected with m1 or m3 receptors produce a rapid agonist-induced Ca^{2+} peak within 15 seconds with calcium returning to near resting levels in 2–3 minutes. Surprisingly, an ACh-induced Ca^{2+} increase was observed even in cells expressing m2 or m4 receptors which couple poorly to PI turnover. Figure 1 shows a typical ACh-induced Ca^{2+} increase for HEK cells transfected with m2 receptors. The Ca^{2+} response in these cells was examined by sequentially imaging only a single line across the field of view thereby obtaining higher time resolution (20 ms). All three cells in the field of view were exposed to 10 μM ACh simultaneously. The Ca^{2+} profiles are similar to m1- and m3-induced responses in that a Ca^{2+} increase occurs rapidly and subsequently decays to near resting levels after several minutes. In this experiment, the Ca^{2+} responses differed from cell to cell in both magnitude and duration. The cell to cell variability in Ca^{2+} responses was present for all mAChR subtypes and could represent a variability in receptor numbers or in any of the components involved prior to Ca^{2+}

release. The cell exhibiting the smallest Ca^{2+} response also showed the broadest initial Ca^{2+} spike and the largest time lag after addition of ACh. Conversely, the largest Ca^{2+} response exhibited a much sharper Ca^{2+} spike and a shorter lag time. The intensity profile of the most responsive single cell is shown in Fig. 1b. The shortest time lag observed for cells transfected with either m1, m2, m3 or m4 receptors, in response to ACh application, was 5–6 seconds. On occasion, the increased Ca^{2+} concentration did not decline steadily but began to oscillate with periods of tens of seconds. The oscillations were present at almost all concentrations of ACh for m2 transfected HEK cells but were seen only at submaximal concentrations for m1 and m3 transfected cells.

An average representation of the speed and magnitude of mAChR Ca^{2+} responses can be obtained for the entire cell population using a flow cytometer and the Ca^{2+} ratioing dye, INDO-1 AM (Molecular Probes) With this technique it is possible to measure the average Ca^{2+} response of thousands of cells per second. Figure 1c shows the average ACh-induced (10 μM) response of m2 transfected HEK cells. The agonist-
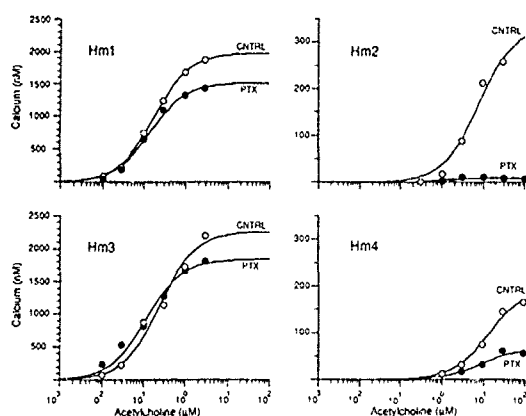
Fig. 2 *Dose–response curves for the peak intracellular Ca²⁺ increases of mAChR subtypes Peak Ca²⁺ was measured with a flow cytometer as described in Fig 1 Open circles show the ACh-induced responses of control cells Closed circles show the ACh-induced cellular responses after PTX treatment (100 ng/ml) for 14–20 hours Each set of data points were fit with a nonlinear regression program to the formula*

$$[\text{peak } Ca^{2+} \text{ response}] = ([ACh] \times B_{max})/(EC50 + [ACh])$$

where B_{max} is taken as the maximum Ca²⁺ response at saturating agonist concentrations and EC50 is the agonist concentration at 50% response All Ca²⁺ measurements were made at room temperature and in physiological saline as described in Fig 1 (approximately 100 000 receptors/cell)

induced Ca²⁺ increase of the entire cell population is similar to the single cell profile presented in Fig. 1b. The Ca²⁺ profiles collected in this manner, however, never clearly exhibited the oscillations observed in single cell imaging, perhaps due to the difficulty in synchronizing the Ca²⁺ responses of an entire cell population. The basic m2 Ca²⁺ profile observed in flow cytometry measurements consisted of a primary Ca²⁺ spike followed by a secondary, smaller and slower Ca²⁺ increase which usually returned to resting levels within 3 minutes of agonist application. A plateau phase was rarely observed. This profile remained virtually unchanged even at higher concentrations of ACh. On the other hand, in cells transfected with m1 or m3 receptors, Ca²⁺ continually declined over the first 2–3 minutes and reached a plateau phase after several minutes. Only at submaximal concentrations of agonist was it possible to separate the Ca²⁺ profile into two spikes for m1 and m3 receptor responses. Apparently, at higher concentrations of ACh in m1 or m3 transfected cells, the secondary Ca²⁺ increase or Ca²⁺ 'shoulder' becomes so prominent that any distinction between the two Ca²⁺ increases becomes obscured. Consequently, the initial decay in Ca²⁺ for m1 or m3 responses appears much slower. These differences in ACh-induced Ca²⁺ profiles could not be attributed to differences in the expression of receptor numbers (see Fig. 3 legend). Additionally, we observed that the plateau phase in m1 or m3 transfected cells appeared to be exclusively dependent on extracellular Ca²⁺. Expos-

ing the cells to zero extracellular Ca²⁺ (nominal Ca²⁺ and 10 mM EGTA) for less than 30 seconds prior to agonist application resulted in a reduced peak Ca²⁺ and a more rapid decline to resting Ca²⁺ concentrations for all muscarinic receptor subtypes.

We obtained ACh dose–response curves for peak Ca²⁺ responses for each of the muscarinic subtypes (Fig. 2). For these data, each mAChR subtype was transfected into chinese hamster ovary (CHO) cells. As reported by Peralta et al.[1] for PI turnover, we found that m1 and m3 receptors exhibited the greatest sensitivity to ACh with comparable EC50s for peak [Ca²⁺] of 170–240 nM while m2 and m4 receptors had EC50s for peak Ca²⁺ of 1 µM and 14 µM respectively (see also Ref. 2). The PTX (100 ng/ml) sensitivity of the receptor subtype responses provides an additional means by which to compare mAChR receptor subtypes. The m1 and m3 receptor responses were the least sensitive to PTX treatment with their peak Ca²⁺ levels never reduced below 75% of control. The m2 and m4 receptor responses, on the other hand, were much more sensitive to PTX treatment; the Ca²⁺ response was completely abolished for CHO cells transfected with m2 receptors (Fig. 2). These data are comparable to those reported by Ashkenazi et al.[3] for the PTX sensitivity of PI turnover. However, intracellular regulatory pathways are complex and it may be that PI turnover is not the only second messenger system capable of stimulating increases in intracellular Ca²⁺ (Fig. 3).

Coupling of muscarinic receptor to a cardiac K⁺ current.

The G protein link between the cardiac M_2 receptor and the atrial muscarinic receptor gated K⁺ channel, $I_{K\ ACh}$, is controversial. By direct application of GTP to the intracellular surface of inside-out patches of atrial membrane, Kurachi et al.[4] demonstrated the GTP dependence of this channel. Direct application of individual purified G protein subunits (for review see Ref. 5) yielded contradictory results. Our lab[6] found that βγ (0.2–1 nM) activated the K⁺ channel while Codina et al.[7] found that α_{40} (called α_K and α_{i3}; 1 pM) activated the channel. Later, we also found that both α_{40} (an α_i) and α_o (10 pM) could activate the $I_{K\ ACh}$ channel but with a much lower success rate (30–50% of intact patches) than βγ activation of $I_{K\ ACh}$ (>90% of patches; Ref. 8). Since βγ was effective only at 20–100-fold higher concentrations than α subunits (>200 pM βγ vs >10 pM α in our hands), some have argued that α is

TABLE I. βγ activation controls

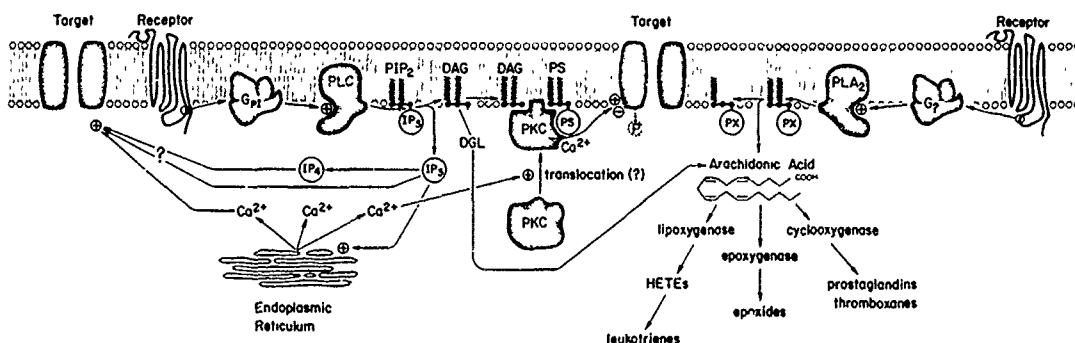| |
|---|
| 1 βγ solubilized in Lubrol or CHAPS activates $I_{K\ ACh}$ |
| 2 Lubrol or CHAPS detergent alone do not activate $I_{K\ ACh}$ |
| 3 Boiled βγ does not activate $I_{K\ ACh}$ |
| 4 Preincubation of βγ with α-GDP prevents βγ-dependent activation of $I_{K\ ACh}$ |

See Refs 12, 14 and 15

Fig 3 Model depicting receptor-coupled G proteins and their subsequent second messenger cascades The left receptor couples to G_{Pi} which activates phospholipase C (PLC) PLC catalyses the splitting of PIP_2 into diacylglycerol (DAG) and inositol trisphosphate (IP_3) DAG activates protein kinase C (PKC) in a phosphatidylserine (PS) and Ca^{2+}-dependent manner PKC can then phosphorylate a target protein, such as an ion channel. IP_3 stimulates the endoplasmic reticulum to release Ca^{2+} The enzyme phospholipase A_2 (PLA_2) is also shown linked to a G protein-coupled receptor (right) This cascade produces arachidonic acid (also produced by diacylglycerol lipase (DGL) from DAG) and is metabolized to other messengers as shown (from Ref 18).

the physiological activator of the channel. This argument is misleading since it assumes equal solubility of subunits and access to the activation site on the channel.

$\beta\gamma$ Activation of $I_{K.ACh}$

Can the $\beta\gamma$ activation of $I_{K.ACh}$, described by our laboratory[6] and Kurachi's[9], be attributed to factors other than the presence of $\beta\gamma$? In brief, it seems unlikely (Table I). Our preparations of $\beta\gamma$ contained less than 0.01% α-subunits (0.1 pM α in 1 nM $\beta\gamma$ subunits), so $\beta\gamma$ activation could not be attributed to α contamination Early controls by our laboratory demonstrated that boiled $\beta\gamma$ and buffer (including CHAPS, the detergent used to suspend the hydrophobic $\beta\gamma$) failed to activate

the channel. In contrast, Kirsch *et al.*[10] reported that CHAPS alone activated $I_{K.ACh}$. This report was puzzling since experiments by both Kurachi's (n>35) and our (n>50) laboratory have not shown CHAPS-dependent activation (Fig. 4). One preparation of transducin $\beta\gamma$, a similar protein, also failed to activate the channel even when suspended in CHAPS[8] (Fig. 4). Furthermore, preincubation with a twofold excess of α_{41}-GDP or a fourfold excess of α_{39}-GDP prevented $\beta\gamma$-dependent activation, presumably by binding free $\beta\gamma$. Finally, addition of α_{41}-GDP to patches preactivated by $\beta\gamma$ turned off $I_{K.ACh}$, consistent with our α preincubation experiments. We were therefore left with the conclusion that the application of $\beta\gamma$, independent of α subunits, is fully capable of activating $I_{K.ACh}$
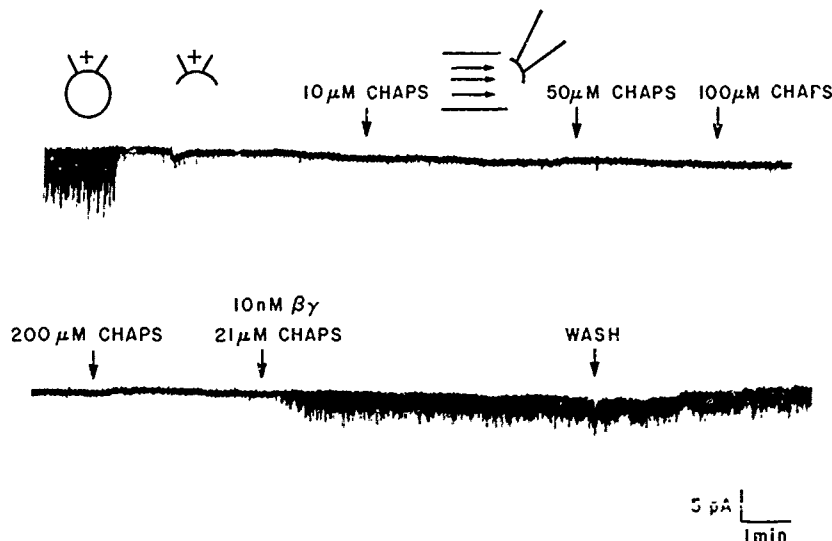

Fig 4 Detergent (CHAPS) does not activate $I_{K.ACh}$ alone An inside-out patch from neonatal rat atria was exposed to various concentrations of CHAPS with no increase in activity Application of $\beta\gamma$ (10 nM) with 21 μM CHAPS rapidly activated the channel (from Ref. 8)

Since application of αGTPγS followed by βγ (and vice versa) did not increase channel activity further, we concluded that both α and βγ activated the same population of channels. Why would the cell use two messengers to modulate one population of channels? An explanation for the puzzle was provided by Kurachi and co-workers who showed that $I_{K\,ACh}$ was activated by arachidonic acid[11]. Since Jelsema and Axelrod[12] had previously shown that transducin βγ activated phospholipase A_2 (PLA_2) in retinal rods, it was logical to test the hypothesis that βγ, acting through PLA_2 stimulation, generated arachidonic acid and its metabolites which in turn activated the channel. Bar-Sagi *et al.*[13] had found an antibody that blocked the generation of lysophospholipids in fibroblasts by inhibiting the function of porcine pancreatic PLA_2. The blockade of βγ (2 nM) activation by the anti-PLA_2 antibody provided the strongest evidence for βγ activation of $I_{K,ACh}$ via PLA_2. Products of the 5-lipoxygenase pathway are most likely involved in activation of the channel[14]. The fact that nordihydroguaiaretic acid and antibody to PLA_2 did not block muscarinic-induced GTP-dependent activation[14] suggests that α can mediate muscarinic-dependent activity. Whether βγ also contributes to muscarinic activation or whether the βγ pathway is used by other receptors is an open question. More decisive evidence for an activator role for βγ has been demonstrated in yeast[15]. Two more general questions are raised by this work. First, do G proteins produce two second messengers, one (βγ) which migrates in the membrane to reach very hydrophobic regions of effectors and a second (α) which interfaces the membrane to more loosely bound or cytoskeletal components of transmembrane proteins? Second, does βγ serve as the activator for PLA_2 in all the systems in which G proteins are thought to interact with PLA_2 (e.g. chemotaxis in neutrophils, mitogenesis in NIH3T3 fibroblasts, exocytosis in mast cells; reviewed by Bourne[16] and Burch[17])? Testing these hypotheses will require more specific methods than simple reconstitution.

Summary

Of the muscarinic receptors, m1 and m3 clearly are linked to PI turnover, apparently via both PTX-sensitive and PTX-insensitive G proteins. The m2 receptor activates $I_{K\,ACh}$ in cardiac atrial and some neuronal cells and inhibits adenylyl cyclase via PTX-sensitive G proteins in many cells. m2 and m4 weakly stimulate PI turnover via PTX-sensitive pathways, but only at agonist concentrations tenfold higher than those needed to stimulate PI by m1 and m3 receptors. Thus, although there are general rules for muscarinic effector coupling, interesting questions remain for future research. Does the weak activation of PI turnover by m2 or m4 receptors mean a loss of specific interaction at the level of the G protein/effector or at the receptor–G protein interface? Can receptors release two activator messengers, α and βγ, and if so, is βγ generally linked to activation of phospholipase A_2?

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G protein-dependent regulation of phospholipase C

C. Peter Downes

A wide variety of receptors for hormones, neurotransmitters and growth factors control cell function via the GTP-dependent activation of phosphoinositide specific phospholipase C (PIC). At least two distinct GTP dependent proteins (G proteins) have been implicated in coupling different receptor populations to the activation of PIC and five immunologically distinct isozymes of PIC have been purified to homogeneity, prompting speculation about the potential for multiple modes of organization of the participants in this signal transduction pathway. The mechanism of hormone and G protein-dependent regulation of PIC has been studied in detail using [³H]inositol labelled turkey erythrocyte membranes and these experiments have provided strong support for the involvement of a heterotrimeric G protein. Further progress requires the development of reconstitution assays in which the regulation of isolated PICs by defined G proteins can be demonstrated.

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) following receptor-dependent activation of phosphoinositide-specific phospholipase C (PIC) simultaneously generates two powerful intracellular signal molecules: inositol 1,4,5-trisphosphate (IP$_3$), which stimulates Ca^{2+} release from intracellular stores; and diacylglycerol, which activates protein kinase C (Ref. 1). Activation of PIC by hormones, neurotransmitters and growth factors is, in many ways, analogous to the hormonal regulation of adenylyl cyclase, but the molecular and mechanistic details of the former are incompletely understood and there is evidence of heterogeneity amongst each of the components of the signal transduction pathway. For example, many receptors (including some of those recognized by muscarinic agonists) are coupled to PIC by G proteins. Based on their differential sensitivity to inhibition by the islet-activating protein of *Bordatella pertussis* (pertussis toxin) at least two distinct G proteins (G$_p$s) have been implicated in the activation of PIC by agonists[2,3]. Protein tyrosine kinase receptors for mitogens, such as epidermal and platelet derived growth factors, are structurally distinct from the G protein-linked receptors whose sequences have been deduced, yet, when activated they stimulate PIC in some cells, but apparently not in others[4–6]. Recent evidence suggests that one form of PIC may be an important substrate for the tyrosine kinase activity of the EGF receptor implicating this covalent modification, rather than a G protein, in the activation of PIC that is observed in intact cells in response to the growth factor[7]. Lastly, it is now apparent that mammalian tissues contain at least 5 immunologically distinct forms of PIC[7]. Whether any of these isozymes can be regulated by G protein-coupled receptors has not yet been established.

How then are the complex, heterogeneous components of the signal transduction pathway leading to PIP$_2$ hydrolysis organized within individual cell types? In this article I will discuss two organizational models which have evolved from analysis of the responses of intact cells to the activation of G protein-coupled

receptors. As discussed below, a number of ambiguities arise in the interpretation of experiments utilizing intact cells. There is an urgent requirement for reliable, broken cell assays of receptor-dependent activation of PIC in which the mechanism, specificity and stoichiometry of the interactions between individual, identified components can be elucidated. Progress made towards this end, using turkey erythrocyte membranes, will also be described.

Organization of signal transduction elements
Convergent coupling mechanisms

The parotid salivary glands of the rat are served by three receptor populations which regulate K$^+$ efflux and water secretion via the activation of PIC and generation of an intracellular Ca^{2+} signal. Inositol phospholipid turnover responses stimulated by maximally effective concentrations of α_1-adrenergic and muscarinic cholinergic agonists or substance P are not additive, which suggests that each receptor is coupled to a common set of signal transduction elements, and the involvement of a pertussis toxin-insensitive G protein has been implicated by studies demonstrating guanine nucleotide dependency of agonist responses in permeabilized cell preparations[8].

This system appears, therefore, to be characterized by the convergence of three receptor populations upon a single G protein and, presumably, a single species of PIC (see Fig. 1). But why are there three receptors that produce identical cellular responses? The truth is that the responses to each receptor are neither quantitatively nor qualitatively identical.

Parotid glands also have β-adrenergic receptors which activate adenylyl cyclase and cause an amylase-rich secretion, so naturally occurring adrenergic agonists (adrenaline/noradrenaline), depending on their concentration, will activate overlapping receptor populations thus affecting the composition of the saliva. By contrast acetylcholine and substance P appear to act only through PIC coupled receptors in parotid gland. The muscarinic receptors do not desensitize appreciably even during prolonged stimulation with maximally effective agonist doses, and substantially elevated levels of intracellular Ca^{2+} are maintained for up to
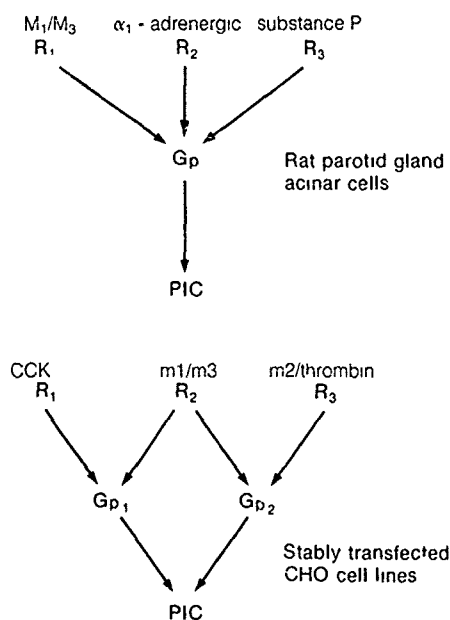
Fig 1. Molecular diversity amongst receptors, G proteins and PIC species could support multiple modes of receptor–effector coupling. Receptor (R) dependent regulation of PIC activity in rat parotid glands is characterized by the convergence of muscarinic cholinergic (M_1/M_3), α_1 adrenergic and substance P receptors upon a pertussis toxin-insensitive G protein and, presumably, a single molecular species of PIC. By contrast CHO cells possess endogenous receptors for thrombin and CCK which regulate PIC via parallel pathways involving distinct pertussis toxin-sensitive (G_{p2}) and insensitive (G_{p1}) G proteins. When they are expressed in these cells, m2 receptors activate PIC via G_{p2} whilst m1 and m3 receptors are capable of divergent coupling through both G_{p1} and G_{p2}, generating pertussis toxin-sensitive and insensitive components of the response to muscarinic agonists[11] Whether these two G proteins regulate the same or different molecular species of PIC is not known at present

30 minutes in a Ca^{2+} containing medium. By contrast substance P receptors desensitize dramatically within about 1 minute of being exposed to the peptide[9]. The response to substance P, therefore, is characterized by rapid initial formation of IP_3 and a Ca^{2+} signal which exceeds that evoked by muscarinic agonists such as carbachol. The maintained response to carbachol, however, greatly exceeds that in response to prolonged exposure to the peptide. Thus, despite their convergent nature, these three receptor populations provide the salivary glands with surprisingly diverse patterns of responsiveness.

Parallel/divergent coupling mechanisms

Receptor-dependent hydrolysis of inositol phospholipids in some cells, notably those of haematopoietic origin such as neutrophils, macrophages and the promyelocytic leukemia cell line, HL60, can be blocked by prior treatment of the cells with pertussis toxin[3]. In

many other cells the response is not blocked by the toxin at concentrations well in excess of those required for ADP-ribosylation of endogenous pertussis toxin substrates[10]. These observations suggest that at least two forms of G_p exist, but that these parallel pathways for the activation of PIC are primarily segregated between different cells and tissues.

More recently pertussis toxin-sensitive and -insensitive forms of G_p have been shown to occur in a homogeneous cell line[11]. Chinese hamster ovary (CHO) cells possess receptors for thrombin and CCK, both of which stimulate inositol phospholipid hydrolysis. Pertussis toxin treatment completely blocked responses to thrombin, but had no effect on the response to CCK. These cells, which lack endogenous muscarinic receptors, were selected by Ashkenazi *et al.*[11] to examine the signal transduction pathways employed by individual muscarinic receptor subtypes following the stable expression of cDNAs encoding m1, m2 or m3 receptors[12]. In the transfected cell lines m2 receptors are efficiently coupled to inhibition of adenylyl cyclase, but apparently more weakly coupled to PIC via a pertussis toxin-sensitive form of G_p. By contrast, activation of m1 and m3 receptors evokes substantial hydrolysis of inositol phospholipids primarily via a toxin-insensitive G_p, but at least partially via the toxin-sensitive G protein. The latter, toxin-sensitive response, was prominent only in cells expressing high levels of muscarinic receptors.

The above studies establish the principle of parallel signalling in a single cell type and also demonstrate that individual receptors may simultaneously activate distinct forms of G_p, or G proteins regulating adenylyl cyclase and PIC, respectively (termed divergent signalling in Fig. 1). Perhaps a more accurate interpretation of these data is that the specificity of a receptor for distinct G proteins in a particular cell is a quantitative, rather than a qualitative property of that receptor. For comparison it is worth noting that endogenous m4 receptors (which are structurally closely related to m2 receptors) are coupled exclusively to adenylyl cyclase inhibition in NG108-15 cells whilst endogenous m3 receptors in 1321N1 cells are exclusively coupled to activation of PIC via a pertussis toxin-insensitive G protein[10,13] It is, therefore, not clear whether divergent coupling mechanisms occur commonly or whether they reflect unusual stoichiometries of signal transduction elements that may occur in transfected cells[12].

The molecular and mechanistic basis of PIC regulation

The analysis of receptor coupling pathways in CHO cells described above relies upon quantitative assessment of the PIC activity in intact cells. This is not readily achieved because of problems such as incomplete inhibition of inositol phosphate dephosphorylation by lithium and variable rates of receptor desensitization. Moreover, the intact cell studies leave open the question of whether distinct G proteins couple to the same or different species of PIC. Clearly, a more complete understanding of the molecular basis of PIC regulation requires the use of broken cell assay procedures and the definitive identification of the G protein and PIC species which participate in the agonist-dependent hydrolysis of PIP_2.

G protein-dependent regulation of PIC

Definitive evidence that a G protein participates in agonist stimulated PIP$_2$ hydrolysis was provided by Cockroft and Gomperts[14] and by Litosch *et al.*[15], who found that guanine nucleotide analogues specifically stimulated the breakdown of endogenous phosphoinositides in membranes of human neutrophils and blowfly salivary glands, respectively. Similar observations have since been made in a large number of membrane preparations and include the important conclusion that agonist responses in these broken cell systems are largely, although not always entirely, guanine nucleotide dependent (see e.g. Ref. 16 for a study involving muscarinic receptors).

It has generally been assumed that G$_p$s are structurally and functionally analogous to the G proteins that regulate adenylyl cyclase (G$_s$ and G$_i$) and cGMP phosphodiesterase (transducin). These proteins, which have been purified to homogeneity and characterized in functional reconstitution assays, are heterotrimers. Their α subunits possess a guanine nucleotide binding site, a high affinity GTPase and are the substrates for ADP-ribosylation by bacterial toxins. They bind GDP in the resting state and the agonist–receptor complex functions by promoting the exchange of GDP for GTP and the dissociation of α_{GTP} from the $\beta\gamma$ complex. In the majority of definitive studies α_{GTP} has been shown to be the active G protein species responsible for effector regulation. Its inactivation is achieved by the intrinsic GTPase, which converts bound GTP to GDP, followed by reassociation with the $\beta\gamma$ complex[17].

In most of the reported studies agonist-stimulated inositol phospholipid hydrolysis in membrane preparations is very much reduced by comparison with the responses of intact cells. By contrast, turkey erythrocyte membranes prepared from [^{3}H]inositol-labelled cells respond to guanine nucleotides with prolonged accumulation of inositol phosphates[18]. The cells are endowed with P$_{2y}$ purinoceptors which remain efficiently coupled to PIC in the membrane preparation and membrane responses to the specific P$_{2y}$ agonist 2-thiomethyl ATP are absolutely dependent on the presence of guanine nucleotides[19,20]. These membranes have, therefore, been used to carry out a detailed analysis of the mechanism of P$_{2y}$ and G protein-dependent activation of PIC.

The activation of turkey erythrocyte PIC by non-hydrolysable analogues of GTP, such as GTPγS, follows first order kinetics and the rate of activation is dramatically accelerated by purinergic agonists[20]. This suggests that agonist-occupied P$_{2y}$ receptors function by accelerating a guanine nucleotide exchange reaction, presumably on G$_p$. Further analogy with known G proteins is suggested by the observations that non-hydrolysable GTP analogues consistently generate a higher level of PIC activity than does GTP itself and that the effects of GTP analogues are reversed by GDP or GDPβS. Inactivation of turkey erythrocyte PIC by the addition of excess GDPβS to membranes that had been preactivated with different GTP analogues revealed that inactivation was very rapid when GTP itself was used to activate, but was extremely slow for non-hydrolysable analogues, particularly so for GTPγS. Taken together these results suggest that an intrinsic

GTPase participates in the inactivation of G$_p$.

The $\beta\gamma$ subunits of the known G proteins are virtually identical to one another and their functions have been the subject of much recent debate. However, the heterotrimeric structure appears to be essential for optimal association of G proteins with activated receptors and subunit reassociation apparently is involved in G protein inactivation[17]. The latter is indicated by experiments showing that reconstitution of purified $\beta\gamma$ subunits with membrane preparations causes inhibition of hormone and/or guanine nucleotide stimulated adenylyl cyclase. Similarly, $\beta\gamma$ subunits injected into *Xenopus* oocytes have recently been reported to inhibit a muscarinic receptor-stimulated chloride current[21], a response attributable to the activation of PIC leading to IP$_3$-stimulated Ca^{2+} release. Reconstitution of $\beta\gamma$ subunits, purified from a variety of sources, with turkey erythrocyte membranes, at concentrations that substantially inhibited adenylyl cyclase also inhibited fluoroaluminate stimulated PIC[22]. By contrast, the same concentrations of $\beta\gamma$ subunits markedly potentiated the activation of PIC by GTP in the presence of a purinergic agonist. These results demonstrate that G$_p$ in these membranes is capable of interacting with the $\beta\gamma$ subunits of conventional G proteins.

Fluoroaluminate is thought to act independently of receptors by imitating the GTP-liganded state of the G protein We have proposed that $\beta\gamma$ subunits inhibit PIC by combining with fluoroaluminate-activated α_p. Although there are other possible interpretations of these results, potentiation of hormone-stimulated PIC could occur if much of the G$_p$ in turkey erythrocyte membranes exists as free α subunits. Combination of
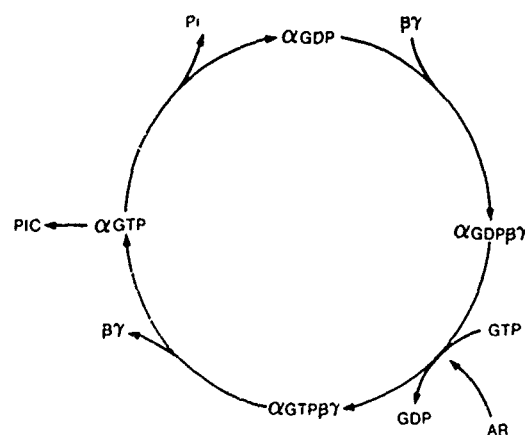
Figure 2 The activation/inactivation cycle of turkey erythrocyte G$_p$. It is proposed that turkey erythrocyte G$_p$ is a conventional, heterotrimeric G protein much of which may exist as GDP-liganded free α subunits in the resting state. Combination of α_{GDP} with endogenous or reconstituted $\beta\gamma$ subunits generates the heterotrimeric form which is necessary for hormonal activation. Agonist occupied P$_{2y}$ receptors (AR) accelerate exchange of GDP for GTP, and dissociation of the GTP-liganded heterotrimer leads to the formation of α_{GTP}, the presumed active G protein species responsible for activation of PIC. Hydrolysis of bound GTP by an endogenous GTPase completes the cycle by reforming α_{GDP}

these with the reconstituted βγ subunits would increase the effective concentration of heterotrimeric G protein which we propose is necessary for effective interaction with activated P_{2y} receptors[22]. A model of the regulation of turkey erythrocyte PIC, based on the above findings, is illustrated in Fig. 2.

Identification of G_ps and G protein-regulated PIC

The experimental results described above provide valuable, but still circumstantial evidence for the involvement of a conventional, heterotrimeric G protein in the regulation of PIC activity. Confirmation that this conclusion is correct requires the isolation of the relevant G proteins and, crucially, direct demonstration of their activity and specificity by reconstitution with either isolated PICs or with PIC-bearing membranes. By this criterion the identities of G proteins involved in receptor-mediated activation of PIC are still unknown. Further indirect evidence suggests that one form of G_i (termed G_{i2}) may be the pertussis toxin-sensitive G protein responsible for coupling chemotactic factor receptors to PIC in human neutrophils[23], but since, as in many cells, receptor-mediated inositol phospholipid hydrolysis is insensitive to both pertussis toxin and cholera toxin, the putative G protein involved is almost certain to be novel.

Isolated membranes which contain prelabelled phosphoinositide substrates and endogenous PIC activity appear, at the present time, to provide the only generally reliable assay systems reflecting the involvement of a G protein in receptor-mediated inositol phospholipid hydrolysis. In order to purify PICs it has been necessary to use exogenous substrates which can be presented as unilamellar vesicles containing pure phospholipid substrates, as mixtures of phospholipids designed to optimize activity or in the presence of ionic detergents such as cholate. Such procedures are ideal for determination of the catalytic activity of PICs, and have enabled the purification to homogeneity of 5 immunologically distinct PIC isozymes[7]. However, such assays may be unsuitable for studying the regulation of PICs because they allow expression of very high levels of enzyme activity. For example, Rhee has pointed out that the total PIC capacity of brain, when assayed using pure PIP_2 as substrate, is sufficient to hydrolyse all of the brain's PIP_2 in 2–20 seconds. However, it seems premature, on this basis, to argue for the existence of negative regulatory proteins[7] whose suppression of PIC activity could be overruled by activators such as G proteins. Ten years ago, Irvine and his colleagues demonstrated that some of the lipid constituents of cell membranes, especially phosphatidylcholine and sphingomyelin, which are abundant in plasma membranes, are powerful inhibitors of PIC activity[24]. Whilst pure phospholipid substrates are rapidly degraded by PICs, phosphatidylcholine-rich unilamellar vesicles or plasma membranes (such as erythrocyte ghosts), containing similar apparent concentrations of PIP_2, are very poor substrates. These considerations do not rule out a role for additional modulatory proteins, but a more parsimonious interpretation of current knowledge would be to suggest that G proteins may activate PICs by

rendering them more capable of utilizing bilayer substrates.

A corollary to the above suggestion is that whilst bilayer substrates may be inappropriate for the sensitive detection of PICs during purification, they are likely to be essential tools for studying their regulation. At present, whether any of the PIC activities that have been purified is responsible for G protein-dependent hormonal stimulation of PIP_2 hydrolysis has yet to be established.

Conclusions

In order to gain further understanding of the organization, regulation and stoichiometry of signal transduction components involved in PIP_2 hydrolysis, molecular genetic techniques must be augmented by seemingly straightforward, yet tantalizingly elusive advances in the biochemical analysis of such systems. Perhaps the most useful step forward would be the development of routine assay procedures which facilitate studies of the regulation of isolated and structurally characterized PIC isozymes by hormone receptors and/or G proteins.

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Regulation of muscarinic acetylcholine receptor function in cardiac cells and in cells expressing cloned receptor genes

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The regulation of the number and function of the muscarinic receptors has been investigated in cultured chick cardiac cells and in cells expressing cloned genes encoding mammalian, Drosophila, and chick muscarinic receptors. A serum-free defined medium for the culture of chick embryonic heart cells has been used to study the regulation of mAChR number and function by serum lipoproteins. Addition of rooster high density lipoprotein to the culture medium results in an attenuation of muscarinic receptor-mediated inhibition of cAMP accumulation without a change in the number of receptors or inhibitory G proteins. Clones encoding the mouse m1 receptor and a homologous receptor from Drosophila have been isolated. When expressed in Y1 adrenal cells, both receptors stimulate phosphoinositide hydrolysis but do not inhibit cAMP accumulation. Deletion of 123 out of the 156 amino acids in the third cytoplasmic loop of the mouse m1 receptor does not impair its ability to stimulate phosphoinositide hydrolysis. A genomic clone encoding a muscarinic receptor expressed in chick heart has been isolated. When expressed in Y1 cells, it causes inhibition of cAMP accumulation but does not stimulate phosphoinositide hydrolysis.

Knowledge of the mechanisms involved in the regulation of the number and functional coupling of neurotransmitter receptors is essential for understanding the molecular basis for signal transduction in the nervous system. This chapter will describe some recent studies on the regulation of the number and function of the muscarinic acetylcholine receptor (mAChR) in chick cardiac cells in culture and the functional properties of cloned mammalian, *Drosophila*, and avian mAChR genes expressed in cultured cells which lack endogenous mAChR.

Muscarinic responses of heart cells cultured in serum-free medium

Cultured embryonic chick heart cells represent an attractive system for the study of the regulation of expression and action of cardiac mAChR *in vitro*. These cells are easy to maintain in primary culture and express high levels of mAChR which can be studied using biochemical and electrophysiological techniques[1]. However, a disadvantage of chick heart cell culture is that the sensitivity and magnitude of mAChR-mediated physiological responses in chick heart cells in culture can be quite variable and dependent on the lot of fetal calf serum (FCS) used. For example, the concentrations of agonist which produce both mAChR-mediated inhibition of adenylate cyclase and stimulation of potassium permeability in cells cultured in medium containing different batches of serum can vary over two-three orders of magnitude[2,3]. To eliminate this source of variability and to provide a system to identify serum and other factors which might

regulate the expression and function of cardiac mAChR, we established a serum-free defined medium for the culture of chick cardiac cells with retention of expression of functional mAChR[4].

Renaud *et al.*[5] reported that chick cardiac cells cultured in medium containing lipoprotein-depleted FCS exhibited an increased mAChR-mediated negative chronotropic response compared to cells cultured in complete FCS. Haigh *et al.*[6] reported that this increased response of chick cells cultured in lipoprotein-deficient serum could be reversed by the addition of bovine low density lipoprotein (LDL). In order to test the role of lipoproteins in the regulation of cardiac mAChR function using a defined homologous system, we tested the effects of purified avian high density lipoprotein (HDL) and LDL on the function of mAChR on chick heart cells cultured in defined medium. Addition of HDL purified from rooster serum to chick heart cells cultured in defined medium causes a decrease in mAChR-mediated inhibition of forskolin-stimulated cAMP accumulation, without changing mAChR-mediated stimulation of phosphoinositide hydrolysis. Addition of rooster LDL also resulted in attenuation of mAChR-mediated inhibition of forskolin-stimulated cAMP accumulation. Addition of HDL to the culture medium did not change the number of mAChR binding sites or their affinity for agonist or antagonist, and did not change the levels of the inhibitory G proteins in the cells. However, addition of HDL decreased the ability of guanine nucleotides to inhibit forskolin-stimulated adenylate cyclase activity in membrane homogenates. Thus, culturing the cells in HDL may interfere with the ability of G proteins to interact with adenylate cyclase. HDL lipid fractions were able to mimic the effect of native HDL in causing both an attenuation of mAChR-mediated inhibition of forskolin-stimulated cAMP accumulation on intact cells and an attenuation of

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guanine nuc<eotide dependent inhibition of k rs, lin-stimulated adenylate cyclase activity in me ' ane homogenate while apoprotein fractions did not cause a decrease in mAChR-mediated inhibition of cAMP accumulation. The effects of HDL and HDL lipid fractions were not correlated with changes in membrane cholesterol content. The results suggest that lipid components of HDL may decrease mAChR-mediated inhibition of adenylate cyclase by decreasing the ability of the inhibitory G protein to interact with adenylate cyclase

Cloning and expression of mammalian muscarinic receptor genes

We used a cDNA clone encoding the porcine m2 mAChR to isolate a genomic clone encoding the mouse m1 receptor[7] The gene encoded a polypeptide of 460 amino acids whose deduced sequence was highly homologous to other m1 mAChRs: there were only thirteen (mostly conservative) amino acid changes from the porcine m1 receptor As with other mammalian mAChR genes, the mouse m1 gene did not contain introns in the coding region of the gene. In order to ensure that this putative mAChR gene encoded a functional receptor, the coding region of the gene was inserted into a mammalian expression vector and transfected into mouse Y1 adrenal and L fibroblast cells. Control experiments demonstrated that in untransfected Y1 or L cells, muscarinic agonists had no significant effect on phosphoinositide hydrolysis or intracellular cAMP levels.The mouse m1 receptor when expressed in Y1 cells exhibited the high affinity binding for quinuclidinyl benzilate, atropine, and pirenzepine expected for an m1 receptor, with dissociation con stants of 78 pM, 0.32 nM, and 10 nM, respectively. The m1 receptor was physiologically active, as evidenced by its ability to activate phospholipase C. In Y1 cells expressing the mouse m1 receptor, carbachol stimulated phosphoinositide hydrolysis a maximum of 20–40-fold. The stimulation in phosphoinositide hydrolysis evoked by 1 mM carbachol could be 90% blocked by 1 µM atropine. Carbachol did not cause an inhibition of cAMP accumulation in these cells; in fact, a slight increase in cAMP levels was observed in response to carbachol. In order to ensure that this apparent selectivity of functional coupling was not due to a defect in the Y1 cells, two types of control experiments were performed. First, the functional responses of the m1 receptor were determined after its expression in a second cell line, mouse L cells. Incubation of transfected L cells with carbachol (1 mM) caused a 2.5-fold increase in phosphoinositide hydrolysis that could be completely blocked by atropine (1 µM). Carbachol did not change the level of cAMP in transfected L cells. Thus, the m1 receptor when expressed in either Y1 or L cells was able to activate phospholipase C but did not inhibit adenylate cyclase.

As a second control experiment, a cDNA clone encoding the porcine m2 receptor was expressed in Y1 cells. The m2 receptor caused inhibition of adenylate cyclase: carbachol caused a 30-65% inhibition in forskolin-stimulated cAMP accumulation. In contrast, carbachol did not cause a significant change in phosphoinositide hydrolysis. Thus, the m1 and m2 re-

ceptors when expressed in Y1 cells evoke different functional responses. Furthermore, these different functional responses show differential sensitivity to ADP-ribosylation of the inhibitory G proteins by islet-activating protein (IAP). Preincubation of Y1 cells with 75 µg/ml IAP completely blocked the ability of the m2 receptor to cause inhibition of forskolin-stimulated cAMP accumulation, but did not inhibit the ability of the m1 receptor to stimulate phosphoinositide hydrolysis. Thus, different subtypes of mAChR when expressed in Y1 adrenal cells mediate different functional responses by interacting with different G proteins

Deletion analysis of functional responsiveness of the m1 receptor

There is considerable evidence that the third putative cytoplasmic loop may be involved in the interaction of such receptors as the adrenergic and muscarinic receptors with specific G proteins[8]. For example, small deletion mutations within this loop in the β-adrenergic receptor cause a loss of receptor-G protein coupling[9,10], and switching this portion of the α- and β-adrenergic receptors switches the physiological response of the chimeric receptor[11]. Similarly, Kubo *et al.* used an oocyte expression system to demonstrate that switching the entire third cytoplasmic loop between the m1 mAChR and the m2 mAChR results in a switch in the type of ion channel coupled to hybrid receptors[12]. Thus, the third cytoplasmic loop appears to contain the amino acid residues which determine the specificity of mAChR coupling.

In order to identify more precisely which portion of the third cytoplasmic loop determines the specific coupling of the m1 mAChR, we constructed deletion mutations in the third cytoplasmic loop of the mouse m1 receptor gene. We then expressed these mutant mAChR in Y1 cells and determined the effect of these mutations on the ability of the mutant receptor to stimulate phosphoinositide metabolism. Two deletion mutants were made, which contain 11 amino acids common to the wild-type receptor on the amino terminal end of the third cytoplasmic loop. The deletions then extend toward the carboxyl terminal and remove either 64 or 123, respectively, of the 156 amino acids in the third cytoplasmic loop. When expressed in Y1 cells, these mutant receptors bound both agonists and antagonists with affinities that were indistinguishable from that of the wild-type receptor. Further, both mutant receptors stimulated phosphoinositide hydrolysis to the same maximal extent and with identical carbachol dose–response curves as the wild-type receptor. These results demonstrate that amino acids 221-343 are not required for coupling of the m1 receptor to activation of phospholipase C, and combined with the results of Kubo *et al.*[12], indicate that the membrane-proximal portions of the third cytoplasmic loop determine the functional specificity of muscarinic receptor action.

Cloning and expression of a *Drosophila* muscarinic receptor

Acetylcholine is a major transmitter in the CNS in *Drosophila*, and mAChR binding sites have been identi-

fied biochemically[13]. Because of the advantages of *Drosophila* which allow a combination of biochemical, behavioral, and molecular genetic studies, we have isolated cDNA and genomic clones encoding a mAChR from *Drosophila*, termed *Dm1*. Analysis of the cDNA clones indicates that they encode a polypeptide of 723 amino acids, considerably longer than any of the previously cloned mammalian mAChR. In contrast to the mammalian mAChR genes, which do not contain introns in the coding regions, the *Drosophila Dm1* gene contains three introns in the portion of the gene encoding the third putative cytoplasmic loop. The deduced amino acid sequence (excluding the highly variable third cytoplasmic loop) is 45–50% identical to the mammalian m1, m2, m3, m4 and m5 receptors. However, if only the membrane-proximal portion of the third cytoplasmic loop is considered, the *Drosophila* mAChR is much more similar to the mammalian m1, m3 and m5 receptors to than the m2 and m4 receptors, suggesting that it would preferentially couple to phosphoinositide hydrolysis rather than inhibition of adenylate cyclase activity.

The cDNA clone encoding the *Drosophila Dm1* receptor was expressed in Y1 mouse adrenal cells to test if it encoded a functional mAChR and to compare its binding and functional properties to that of the mammalian mAChR previously expressed in this cell line. The expressed *Dm1* gene product exhibited high affinities for the muscarinic antagonists quinuclidinyl benzilate and atropine (dissociation constants of 86 pM and 0.82 nM), confirming that it encoded a muscarinic receptor Furthermore, these values are consistent with those reported by Haim *et al.*[13] for the binding of muscarinic ligands to receptors in homogenates prepared from *Drosophila* heads.

The relatively high homology of the *Dm1* receptor to the mammalian m1, m3 and m5 receptors in the membrane-proximal portions of the third cytoplasmic loop raises the possibility that the *Drosophila* receptor would be functionally homologous to these receptors and preferentially couple to stimulation of phospholipase C rather than to inhibition of adenylate cyclase. Incubation of transfected Y1 cells with carbachol increased phosphoinositide hydrolysis 18–20-fold over control levels. As was found previously for the mouse m1 receptor expressed in Y1 cells, carbachol did not cause a decrease, and in fact caused an increase, in the level of forskolin-stimulated cAMP accumulation in intact cells. Thus, the *Drosophila Dm1* mAChR is both structurally and functionally homologous to the mammalian m1 mAChR.

The isolation of the *Drosophila* mAChR gene will allow a genetic dissection of the role of this receptor in the whole animal. The introduction of wild-type and mutant mAChR genes into *Drosophila* containing deletions of the locus encoding the *Dm1* gene combined with behavioral and biochemical studies should provide valuable information on the physiological significance and functional role of the mAChR in *Drosophila*.

Cloning and expression of an avian muscarinic receptor

The embryonic chick heart has been used extensively as a model system for the study of the mechanisms

involved in the regulation and development of mAChR number and function in cardiac tissue[1] While there are many similarities between mAChR in mammalian and chick hearts, there are also a number of significant differences. The affinities of the two types of receptor for the M[1]-selective antagonist pirenzepine differ dramatically, with chick heart mAChR having a significantly higher affinity for pirenzepine than mammalian cardiac mAChR[14]. The receptors can also be distinguished immunologically: a number of monoclonal antibodies which recognize mAChR from hearts of a variety of mammalian species do not crossreact with the chick heart mAChR[15]. In addition, peptide maps of mAChR from mammalian and chick hearts are different[16]. We cloned the chick heart mAChR in order to determine the molecular basis of the differences between mammalian and avian hearts and to have a molecular probe for the study of the regulation of mAChR gene expression in the chick heart.

A chicken genomic library was screened with a mixture of nick-translated probes encoding the mammalian m1 and m2 receptors. One strongly hybridizing clone was selected for further study and shown to hybridize a 2.8 kb RNA expressed in chick brain and heart. Sequence analysis demonstrated that this gene encoded a polypeptide of 490 amino acids which had a high degree of homology to the mammalian m2 and m4 receptors. When expressed in Chinese hamster ovary cells, this mAChR exhibited affinities for quinuclidinyl benzilate and pirenzepine of 110 pM and 55 nM, respectively, similar to the values previously reported for the mAChR in chick heart[14,16]. In order to determine the functional specificity of this chicken mAChR, it was expressed in Y1 cells. Incubation of the cells with carbachol caused a 35% inhibition of forskolin-sensitive cAMP in intact cells; there was no stimulation of phosphoinositide hydrolysis in transfected Y1 cells following incubation with carbachol Thus. the chick heart mAChR has a high degree of both sequence and functional homology to the mammalian cardiac mAChR.

Conclusions

The ability to culture chick heart cells in defined, serum-free medium should provide a useful system for the identification of factors and hormones which regulate mAChR number and function in the heart. The combination of biochemical, physiological, molecular biological, and genetic approaches should provide a wealth of information on the mechanisms of action and regulation of the muscarinic receptor.

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Muscarinic activation of potassium channels in cardiac myocytes: kinetic aspects of G protein function *in vivo*

*Gabor Szabo and Angela S. Otero

Muscarinic agonists open potassium-selective $K_{(ACh)}$ channels in cardiac myocytes of pacemaker or atrial origin. Receptor activation is coupled to channel opening by a membrane bound guanine nucleotide-binding protein (G_K) through a process that does not require cytoplasmic intermediates. We have used the muscarinic potassium channel and the corresponding macroscopic current, I_{ACh}, as rapid, sensitive and specific indicators of the state of activation of G_K. This approach, developed here in quantitative detail, allowed us to identify the salient kinetic processes involved in the activation and deactivation of G_K in vivo. Agonist was found to act by accelerating the rate of GDP release, and the subsequent GTP uptake by G_K, while deactivation was found to occur by a process that requires GTP hydrolysis. Unexpectedly, deactivation in the intact system is much more rapid than the rate of GTP hydrolysis by isolated G proteins, suggesting the presence of a GTPase-stimulating factor in intact cells.

One of the first observed effects of acetylcholine, the slowing of the heart rate[1], can be attributed mostly to the opening of potassium-selective, ion-conductive channels in the cardiac pacemaker and atrial cell membrane in response to the activation of muscarinic receptors[2]. A significant, steeply temperature dependent delay ($\approx$100 ms) between receptor activation and channel opening has been the first indication that occupation of receptors by agonist is coupled to channel opening by a multistep process[3,4]. The involvement of diffusible, cytoplasmic intermediates has been ruled out, however, by experiments in which channel activity was monitored in an isolated patch of the cell membrane: agonist applied to the isolated patch activated channels within the patch while agonist applied outside the patch, a maneuver that should have generated a large amount of cytoplasmic messenger,

did not[5]. The involvement of membrane bound guanine nucleotide binding proteins (G proteins)[6,7] in the coupling process has been demonstrated by the observation that intracellular application of poorly hydrolysable analogs of GTP leads to persistent, antagonist-resistant channel activation and also by the fact that pertussis toxin, which is known to inactivate a class of G proteins by ADP ribosylation, abolished channel activation by agonist[8,9]. These original observations have been confirmed and extended using single channel measurements in excised patches of cell membranes[10,11]. It has been shown, in particular, that purified G_i-type G proteins activated by GTPγS and applied to the intracellular aspect of excised atrial cell membranes, can activate K+ channels directly[12,13].

Channel conductance as an indicator of G_K activation

Direct activation of the K+ channel by purified G protein in patches suggests the possibility of using the macroscopic current through the muscarinic activated K+ channels, I_{ACh}, as an indicator of the degree of activation of G_K, the G protein that opens the channel *in vivo*. Single channel measurements using cell-

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attached patches of cell membrane indicate that application of agonist to the extracellular aspect of the cell membrane increases the rate of channel opening but does not affect single channel conductance or mean channel open time[5,10,14]. Moreover, the probability of channel opening remains small even at maximally activating concentrations of agonist This is illustrated in Fig 1 for an experiment performed in the on-cell configuration. Single channel openings (upward deflections) were induced by the perfusion of a patch pipette with 1 μM ACh. Note that there are at least three $K_{(ACh)}$ channels in the patch since the simultaneous opening of three channels is observed on at least one occasion. Most of the time, however, only one channel is open, indicating that the probability of the channel being open is much less than unity even at maximal activation of the system. At lower concentrations of ACh, channel open probability decreases proportionately. Taken together, these data suggest that the channel open probability may be used to assess the degree of G_K activation:

$$p_o = \gamma \Gamma^* \qquad (1)$$

where p_o is the channel open probability, Γ^* is the membrane concentration of activated G_K and γ is a factor of proportionality. The overall current I_{ACh} for a cell having N muscarinic activated K^+ channels is given by.

$$I_{ACh} = N p_o I_{SC} = N\gamma I_{SC}\Gamma^* \qquad (2)$$

where I_{SC} is the single channel current for a given potential applied across the membrane. The maximal current is attained when all of the G_K available in the membrane, G_{tot}, is activated:

$$I_{ACh}^{max} = N\gamma I_{SC}\Gamma_{tot} \qquad (3)$$

so that the fraction of the current activated under a given experimental situation is a direct measure of the fraction of G_K activated:

$$\frac{I_{ACh}}{I_{ACh}^{max}} = \frac{\Gamma^*}{\Gamma_{tot}} \qquad (4)$$

The simplest model for the activation of G proteins

consists of two states, namely the resting, diphosphate bound state and the activated, triphosphate bound state. At present, the most successful experimental approach to study activation in the absence of agonist is to flood the cell with a hydrolysis resistant analog of GTP, denoted here as GXP. Since nucleotide hydrolysis and therefore G protein deactivation is not possible, as shown in the following scheme:

$$\text{Resting} \xrightarrow{k_m} \text{Activated}$$
$$\text{state} \qquad \text{state}$$
$$(G_{GDP}) \qquad (G^*_{GXP})$$
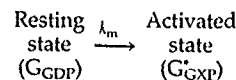
in the absence of agonist, release of GXP from G^*_{GXP} is very slow and G_K will accumulate in its activated form. The rate k_m at which this process occurs under various experimental circumstances reveals much about the mechanism of G protein activation. Noting that:

$$\Gamma + \Gamma^* = \Gamma_{tot} \qquad (5)$$

where Γ is the membrane concentration of non activated, GDP bound G_K, the rate of increase in the membrane concentration of the activated, GXP bound form of G_K is given by:

$$\frac{d\Gamma^*}{dt} = k_m\Gamma = k_m(\Gamma_{tot} - \Gamma^*) \qquad (6)$$

which can be integrated to yield:

$$\Gamma^*_{(t)} = \Gamma_{tot}(1 - e^{-k_m t}) \qquad (7)$$

It is found, in particular, that the initial rate at which the fraction of activated G_K increases is a direct measure of k_m.

$$k_m = \frac{d}{dt}\left(\frac{\Gamma^*}{\Gamma_{tot}}\right)_{t=0} \qquad (8)$$

Considering (Eqn 4), it also follows that the initial fractional rate of appearance of I_{ACh} is actually equal to the rate constant k_m.

$$\frac{d}{dt}\left(\frac{I_{ACh}}{I_{ACh}^{max}}\right)_{t=0} = \frac{d}{dt}\left(\frac{\Gamma^*}{\Gamma_{tot}}\right)_{t=0} = k_m \qquad (9)$$

Fig 1 The effect of local application of 1μM ACh to a patch of atrial cell membrane The patch pipette, perfused in the cell-attached configuration, was filled with 100 mM KCl, 25 mM HEPES (pH 7 4) and held at 0 mV The resting membrane potential is near −85 mV ; Arrow marks the start of perfusion
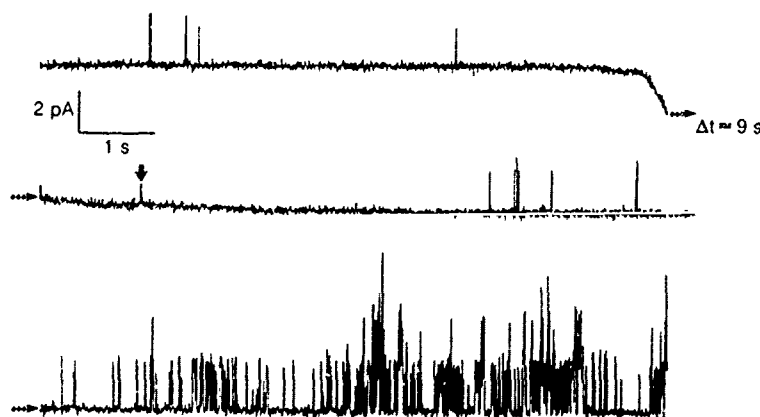
Rate constants of receptor-independent and receptor-dependent activation of G_K

We have previously characterized initial rates of activation for I_{ACh} observed following the injection of several hydrolysis-resistant analogs of GTP into atrial myocytes[15]. The preceding analysis demonstrates that the initial rates in fact correspond to the actual rate constants of G_K activation. In the absence of agonist, when myocytes are rapidly flooded with hydrolysis-resistant analogs having different structures (guanosine-5'-O-[3-thiotriphosphate], GTPγS, guanylylimidodiphosphate, GppNHp; and guanylyl[β,γ-methylene]-diphosphate, GppCH2p), the rate constants of receptor-independent activation of G_K were found to be essentially the same, near 0.4 min^{-1}. An identical rate constant for the uptake of structurally different analogs suggests that G_K activation is limited by a process that precedes triphosphate binding; this process is likely to be the receptor-independent, spontaneous release of GDP from G_K. If so, then the rate constant for basal GDP release by G_K in cardiac myocytes is 0.4 min^{-1}.

On application of agonist, the rate constant of activation increases linearly with agonist concentration in a manner that is independent of the structure of the GTP analog, suggesting again that agonist acts by increasing the rate of GDP release from G_K. For ACh, the rate constant of GDP release is estimated this way to be 8.4×10^8 [ACh] in units of min^{-1} M^{-1}. Note that there is a specificity among the three GTP analogs for the nucleotide binding site of G_K. This becomes evident when significant concentrations of GTP are present and compete for the nucleotide binding site. As G_K activated by GTP is rapidly deactivated owing to hydrolysis, GTP effectively reduces the rate at which the analog is binding to and persistently activates G_K. The order of decreasing effectiveness for the analogs to compete with GTP is GTPγS > GTP > GppNHp > GppCH2p. A specificity in the ability of GTP to shunt persistent activation by analogs implies the existence of a transient state of G_K in which nucleoside triphosphate exchange may occur rapidly and come to equilibrium within the time scale of GDP release.

Rate constant of G_K deactivation

Under physiological circumstances, I_{ACh} decays rapidly upon removal of the agonist, with $t_1 < 300$ ms for atrial myocytes. Under the simplest circumstances, the process of deactivation may be described as a first order rate process with rate constant k_{cat}. The activation-deactivation cycle may be described by the following scheme:

$$G_{GDP} \underset{k_{cat}}{\overset{k_m}{\rightleftharpoons}} G^*_{GTP}$$
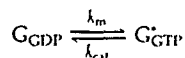
while the corresponding rate equation can be written as

$$\frac{d\Gamma^*}{dt} = k_m \Gamma - k_{cat} \Gamma^* \tag{10}$$

On removal of agonist, k_m falls to a negligibly small value. Equation 10 has the following solution for this case:

$$\Gamma^* = \Gamma_{tot} \frac{k_m^U}{k_m + k_{cat}} e^{-k_{cat}t} \tag{11}$$

The rate constant of deactivation can therefore be calculated from the decay time of I_{ACh}, which is equal to that of G*. For atrial myocytes, the observed t_1 of < 300 ms corresponds to $k_{cat} > 200$ min^{-1}. A similar result is obtained by extrapolating the rate of activation determined with the help of hydrolysis-resistant analogs, to the half-activating ACh concentration of the I_{ACh}–[ACh] dose–response curve, 160 nM. At this concentration of ACh, $k_{cat} = k_m$ and $k_m = 8.4 \times 10^8 \times 1.6 \times 10^{-7} = 134$ min^{-1}. Both of these estimates are nearly two orders of magnitude faster than rates of GTP hydrolysis by purified G proteins, which are in the range of 4 min^{-1}.

There are at least two explanations for this discrepancy. It is possible that G_K is not deactivated by GTP hydrolysis but by GTP–GDP nucleotide exchange instead. A more likely possibility is that the GTPase activity of G_K is increased *in vivo* by the presence of an additional component in the membrane. Recent experiments indicate that in the presence of activating concentrations of agonist, nucleotide exchange may occur rapidly[16]. Thus, extracellular application of a pulse of agonist to cells in which I_{ACh} has been activated in a receptor-independent manner by intracellular perfusion of a mixture of a poorly hydrolysable GTP analog and GTP, markedly reduces or abolishes I_{ACh}. This indicates that agonist induces rapid nucleotide exchange at the previously occluded nucleotide binding site[16]. Note, however, that the presence of GTP is obligatory for this phenomenon to be observed; when only GDPβS and GXP are present, the agonist-induced deactivation is absent. Thus, while nucleotide exchange is possible in the presence of agonist, GTP–GDP exchange cannot account for deactivation of G_K on removal of agonist. These observations argue strongly for GTP hydrolysis being responsible for deactivation. Note, however, that GTP hydrolysis by purified G proteins is much slower than the rate of G protein deactivation *in vivo*. The discrepancy suggests that intact cells may contain a factor that accelerates GTP hydrolysis by G_K. This may be the effector itself (e.g. the channel protein) or some unidentified factor analogous to GAP, the cytosolic protein that stimulates the GTPase activity of p21^{ras} protein[17].

A kinetic scheme for G protein function

The scheme (Fig. 2) summarizes and attempts to integrate presently available structural and kinetic information concerning the detailed mechanisms by which G proteins couple the signal of receptor activation to the modulation of effector function. The left-hand 'primary' cycle, which is likely to operate under normal physiological circumstances, is composed of the resting, heterotrimeric form of G_K which, upon interaction with activated receptor (R*), dissociates into α and $\beta\gamma$ subunits. The rate of this process is governed by the concentration of receptors activated by agonist, giving rise to the agonist dependence of this
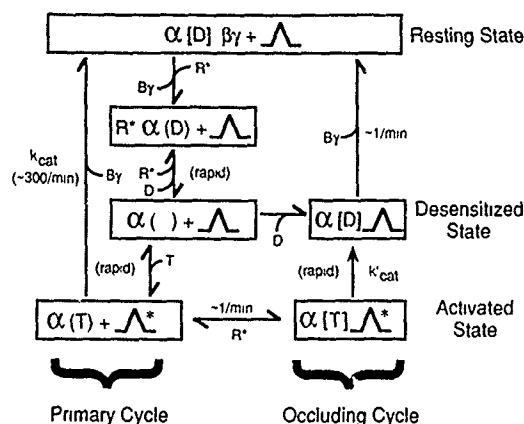
Fig 2 Kinetic scheme for the signal coupling function of G proteins α, β and γ G protein subunits [ ] occluded nucleoside binding site ( ) deoccluded nucleoside binding site D nucleoside diphosphate, T nucleoside triphosphate The open state of the K_{ACh} channel is indicated by asterisks

process. Activated receptor induces an open, nucleoside triphosphate-preferring configuration of the binding site on the α subunit which, under physiological conditions, causes the release of GDP and uptake of GTP. In its nucleoside triphosphate bound form, the α subunit interacts with the channel and increases the probability of its opening. Although GTP hydrolysis usually returns the system to its resting state, the 'open' activated state may also be converted into an occluded state in which nucleotide at the binding site cannot exchange with that in the surrounding medium. The process of occlusion is best observed in the presence of poorly hydrolysable GTP analogs which prevent the return to the resting state; we have observed a rate of occlusion near 1 min^{-1} at subthreshold concentrations of agonist under these circumstances. Note that the channel activity induced by GTPγS-bound α subunit is not sensitive to inactivation by functional antibodies raised against the α subunit, implying that channel and α subunit form a stable complex in the occluded form[18]. At high concentrations of agonist, activated receptors favor deocclusion, again leading to the appearance of an 'open' form[16,19]. GTP

hydrolysis is also possible in the occluded active state. However, the product of this process is a hypothetical 'desensitized' state in which deactivated α subunits having GDP occluded in the binding site form a stable complex with the channel. A relatively slow buildup of this inactive state would reduce the number of channels available for activation and may explain the rapid phase of the desensitization seen on prolonged application of agonist[20]. By a slow exchange of channel for βγ subunits, the desensitized form can return in our scheme to the resting state, accounting for the slow recovery of the system from desensitization. While the scheme of Fig. 2 is consistent with the majority of the observations made on G protein mediated channel activation, further experiments will be required to ascertain its validity in detail.

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Structure–activity relationships and pharmacological profile of selective tricyclic antimuscarinics

W.G. Eberlein, W. Engel, G. Mihm, K. Rudolf, B. Wetzel, M. Entzeroth, N. Mayer and H.N. Doods

The discovery of the M_1-selective receptor antagonist pirenzepine was the impetus for a research project directed towards the development of selective muscarinic antagonists In the pursuit of this objective, compounds with different selectivity profiles have been found. AF-DX 116 was the first cardioselective antagonist synthesized. Subsequently novel M_2 receptor antagonists have been discovered with higher potency and selectivity. Moreover, a pirenzepine-type compound UH-AH 37 has been identified that, in contrast to pirenzepine, shows a higher affinity for ileal than for atrial muscarinic receptors. Among tricyclic muscarinic receptor antagonists three different selectivity profiles have been identified, namely: $M_1 > M_3 > M_2$, M_{sm} for pirenzepine; $M_2 > M_1 > M_3$, M_{sm} for AF-DX 116, AF-DX 384, AQ-RA 741; and $M_{sm} \approx M_1 > M_2$, M_3 for UH-AH 37 and its (+) enantiomer.

The tricyclic muscarinic receptor antagonists pirenzepine and AF-DX 116 were respectively the first M_1 and M_2 selective drugs intensively used as research tools. In this paper we summarize part of our work directed towards the development of selective tricyclic muscarinic receptor antagonists with greater or different selectivity

Tricyclic compounds as selective antimuscarinics

The structure of pirenzepine is depicted in Fig. 1. It consists of a butterfly-shaped tricyclic ring system and a (methylpiperazinyl)-acetyl side-chain, which are connected by a carboxamide bond. It is well known from receptor binding studies that pirenzepine exhibits substantial affinity only towards the muscarinic receptor system[1]. Attempts to interpret these findings have recently been undertaken both by semiempirical quantum mechanical calculations[2] and by the establishment of structure–activity relationships[3]. In the case of pirenzepine, the differences in binding affinities between the muscarinic receptor and for example the imipramine receptor are brought about by three features: the endocyclic amido group, the position of the protonated nitrogen with respect to the tricyclic ring and to a minor extent the exocyclic amide bond (see left panel of Fig. 2) A detailed analysis[4] of the muscarinic antagonists of the pirenzepine type revealed important features for M_1 selectivity as summarized on the right in Fig. 2. M_1 selectivity is controlled by both the tricycle and the side-chain but with a dominant influence of the side-chain. Among the tricycles studied, highest selectivity has been obtained with compounds containing the pyridobenzo-diazepinone system. As to the influence of the side-chain on M_1 selectivity it has been found that semirigid

systems with six-membered rings, which obviously force the protonated nitrogen into a definite spatial orientation in relation to the tricycle, are especially favourable.

In the course of this systematic variation of the side-chain of pirenzepine we discovered compounds with a different selectivity profile. These exhibited high affinity for the receptors of the heart. The most promising member of this family of M_2-selective compounds is represented by AF-DX 116 (Ref. 5; Fig 3). It is important to note that structural differences between pirenzepine and AF-DX 116 are very subtle. The side-chain has been modified in such a way, that the most basic nitrogen is not incorporated into the ring itself but is attached to it via a methylene bridge. In contrast to pirenzepine, AF-DX 116 is able to discriminate clearly between the cardiac and the glandular muscarinic receptors.

As part of our research strategy we decided to look for follow-up compounds with two objectives in mind We felt it might be possible to identify compounds which are more potent and which possess an increased heart/smooth muscle selectivity. But how could more pronounced M_2 selectivity be achieved? The line we followed was based on structure–activity relationships already established. The tricycle was considered to be
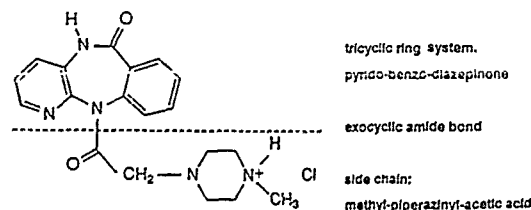
Department of Pharma Research, Dr Karl Thomae GmbH, Biberach an der Riss, FRG.

tricyclic ring system.
pyrido-benzo-diazepinone

exocyclic amide bond

side chain:
methyl-piperazinyl-acetic acid

Fig 1 Structure of pirenzepine

only of minor influence. However, a side-chain with higher flexibility seemed to be essential. Based on the structural differences of sets of M_1- and M_2-selective compounds, we postulated that M_1 and M_2 selectivity is controlled by different spatial orientations of the protonated side-chain nitrogen. This hypothesis could be substantiated by extensive conformational analyses with molecular mechanical calculations.

Based on these ideas a systematic variation of the structural elements[6] of the side-chain of AF-DX 116 has been performed as shown in Figs 4 and 5. Our strategy proved to be very successful because two interesting compounds could be identified within the series depicted in Figs 4 and 5. The first compound, AF-DX 384, is the result of the synthesis of a series of urea analogs of AF-DX 116. It shows improved activity and selectivity similar to AF-DX 116. The most attractive derivative of the series shown in Fig. 5 represents compound AQ-RA 741 Compared to AF-DX 116 it shows tenfold higher activity and improved heart smooth muscle selectivity. Furthermore AQ-RA 741 has the advantage of being achiral.
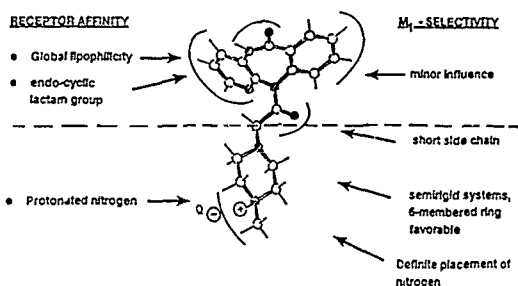

AF-DX 116   UH-AH 37

Fig 3 Chemical structures of AF-DX 116 and UH-AH 37

compared to atrial muscarinic receptors. Thus, UH-AH 37 and its enantiomer UH-AH 371 represent interesting tools for further elucidation of the muscarinic system.

Biological properties of select.ve tricyclic muscarinic receptor antagonists

Pirenzepine

Pirenzepine binds only to M_1 receptors with high affinity, whereas compounds like dicyclomine, 4-DAMP and hexahydro-sila-difenidol (HHSiD) do not exhibit this selectivity profile[9]. The affinity of pirenzepine for M_1 receptors varies between 4 and 20 nmol/l depending on the test system used. We have examined the selectivity profile of pirenzepine in detail in both *in-vitro* and *in-vivo* models. The pK_i values of pirenzepine for cortical M_1, cardiac M_2, glandular M_3 and ileal smooth muscle binding sites are 8.0, 6.3, 6.8 and 6.4, respectively. In functional studies muscarinic agonist effects on inhibitory M_1 receptors in rabbit vas

Fig 2 Summary of structure–activity and selectivity relationships of M_1-selective compounds

Another interesting issue for drug design within the class of tricyclic compounds refers to the principles of chirality of the tricyclic ring As shown in Fig. 6 the tricyclic system might exist as a pair of stable enantiomers provided that the barrier to inversion is sufficiently high[7]. In the case of pirenzepine for example the barrier to inversion appears to be too low for separation of the enantiomers at room temperature. The investigation of a large set of pirenzepine-like aminoacylated tricyclic compounds has demonstrated that the barrier to inversion is extremely sensitive to the size of substituents in the peri-position adjacent to the basic side-chain[8]. This feature made compound UH-AH 37 (Fig. 3) a likely candidate for separation since it carries a bulky chlorine atom in one of the critical peri-positions. In fact, we found that UH-AH 37 is a racemate which can easily be separated into its two enantiomers. The (+) enantiomer of this compound UH-AH 371 is much more potent than the (--) isomer.

In pharmacological experiments UH-AH 37 and its (+) enantiomer showed another interesting feature. Although UH-AH 37 and UH-AH 371 reveal a binding profile similar to pirenzepine, in functional tests both compounds possess a tenfold higher affinity for ileal
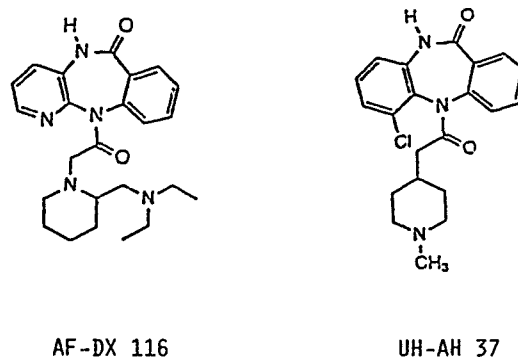

ACTIVITY   : AF-DX 384 > AF-DX 116
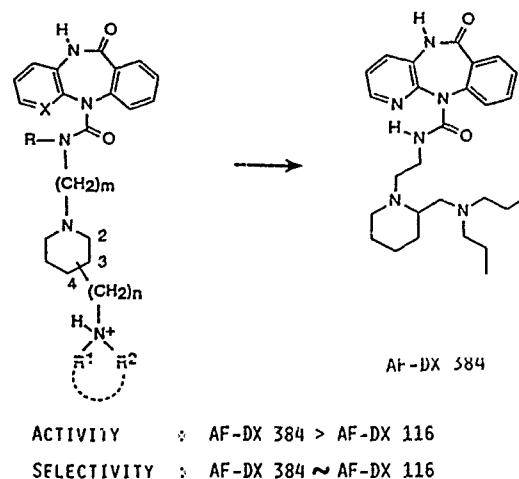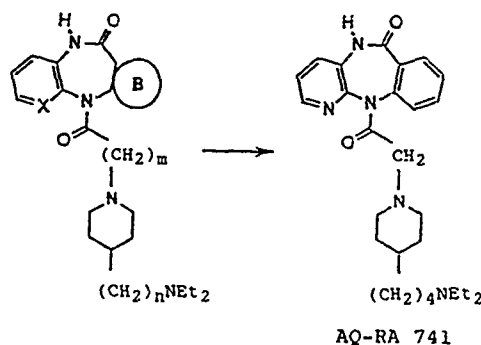
SELECTIVITY : AF-DX 384 ~ AF-DX 116

Fig 4 General formula of urea analogs of AF-DX 116 and structure of AF-DX 384

AQ-RA 741

ACTIVITY : AQ-RA 741 > AF-DX 116

SELECTIVITY : AQ-RA 741 > AF-DX 116

Fig 5. General formula of AF-DX 116 analogs substituted in the 4 position of the piperidine ring

deferens, guinea-pig cardiac M_2 receptors regulating heart rate, rat pancreatic M_3 receptors mediating α-amylase secretion and guinea-pig ileal receptors responsible for smooth muscle contraction (denoted by M_{sm} in this paper) were antagonized with pA_2 values that correspond to 8.2, 6.8, 6.5 and 6.7, respectively. Accordingly, the M_1 selectivity of pirenzepine amounts to approximately 16–79-fold in comparison to the other subtypes. In the rat pirenzepine exhibits a marked selectivity. Almost 22-fold lower doses are sufficient to antagonize the McN-A-343 induced increase in blood pressure (ED50 = 112 nmol/kg, i.v.) than the bradycardia induced by vagal stimulation. The increase in blood pressure is due to stimulation of M_1 receptors in sympathetic ganglia which results in the release of noradrenaline. Pirenzepine also possesses selectivity for M_1 receptors compared to those responsible for the pilocarpine evoked salivation (sixfold). Moreover, in the lumen-perfused stomach preparation of the urethane-anaesthetized rat, a dose of 62 nmol/kg produces 50% suppression of the acid secretion induced by electrical stimulation of vagal fibers. Approximately 40-fold higher doses of pirenzepine are necessary to

inhibit the vagally mediated bradycardia in the rat (ED50 = 2512 nmol/kg). The selectivity profile, therefore, is $M_1 > M_3 > M_2$, M_{sm}.

AF-DX 116, AF-DX 384 and AQ-RA 741

The interaction of AF-DX 116 with the M_2 receptor is characterized by a dissociation constant of about 1×10^{-7} M. The M_1 receptors as well as those on intestinal smooth muscle exhibit an intermediate affinity for AF-DX 116. The glandular M_3 type has a low affinity for AF-DX 116 with a pK_i value of 5.7.

Thus, unlike pirenzepine, AF-DX 116 is able to discriminate markedly between cardiac and glandular muscarinic receptors. This approximately 30-fold difference in affinity aided the classification of cardiac and
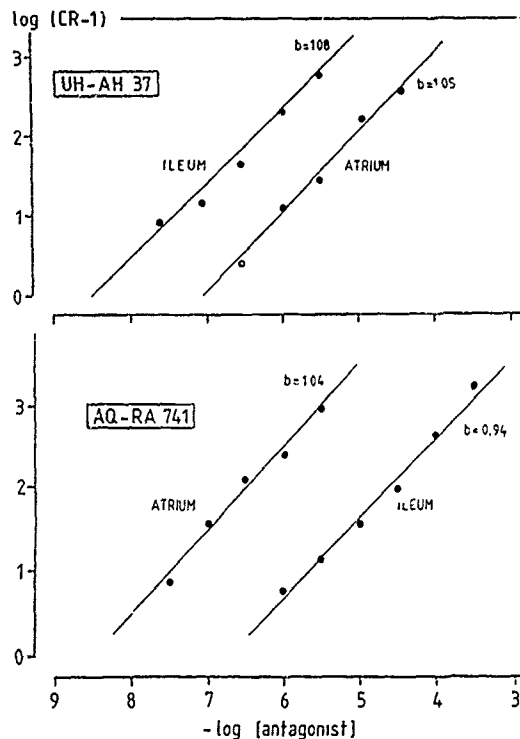
Fig 7 Arunlakshana-Schild plots of the antagonism by UH-AH 37 and AQ-RA 741 of the responses to methacholine at the muscarinic receptors mediating ileal contraction and heart rate
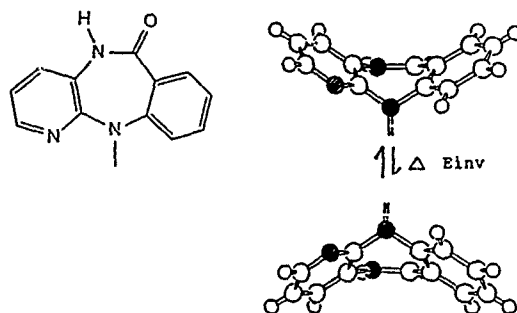
Fig 6. Chemical structure of the pyridobenzodiazepine system and atropisomerism of the tricycle

glandular muscarinic receptors into M_2 and M_3, respectively[9]. The in-vivo selectivity of AF-DX 116 in rats is in agreement with in-vitro data. The vagally induced bradycardia is blocked by AF-DX 116 in doses 26- and 6-fold lower than those needed to antagonize agonist-induced salivation and increase in blood pressure, respectively (see Table I). Phase I studies show that after i.v. or oral administration of AF-DX 116 heart rate is increased without significant changes in salivary flow, pupil diameter and near-point vision[10].

In the course of the search for successors of AF-DX

116 two new compounds (AQ-RA 741 and AF-DX 384) with interesting properties have been found. AF-DX 384 exhibits a higher affinity for muscarinic receptors compared to AF-DX 116 (see Table I) with a comparable degree of selectivity. AQ-RA 741 is an AF-DX 116 analog with a 15–30-fold higher affinity for cardiac M_2 receptors. In addition, the cardiac versus ileum selectivity of this compound ($\approx$ 80-fold) is more pronounced whereas the M_2/M_3 ratio is unchanged (Table I, Fig. 7) To date this drug is the most potent M_2 antagonist with a selectivity profile comparable to that of methoctramine. In pithed rats AQ-RA 741 (i.v.) is sevenfold more potent than AF-DX 116 in antagonizing the bradycardia elicited by stimulation of the right vagus nerve. In addition the cardioselectivity is more pronounced (Table I). The *in-vivo* selectivity of AQ-RA 741 in the anaesthetized cat is shown in Fig. 8. In this model the administration of methacholine (50 μg i.v. and i.a.) results in a decrease in heart rate, increase in duodenal and bladder pressure, bronchoconstriction, and enhanced salivation. The –log ED50 value (mol/kg) of AQ-RA 741 to antagonize the bradycardia was 7.53, whereas 13–67-fold higher doses were necessary to antagonize the effects on duodenal pressure (–log ED50 = 6.03), bladder pressure (–log ED50 = 5.94), airway resistance (–log ED50 = 6.40) and salivation (–log ED50 = 5.70). Thus the selectivity profile of AF-DX 116, AF-DX 384 and AQ-RA 741 is: $M_2 > M_1 > M_3$, M_{sm}.

The direct effects of the parasympathetic nervous system on the cardiac function are a reduction in heart rate and a slowing of the rate of conductance through the AV node. Because the receptors mediating these effects are of the M_2 subtype, it is anticipated that M_2-selective antagonists like AF-DX 116 and AQ-RA 741 will be useful in the treatment of bradycardic disorders and certain types of AV block[11].

UH-AH 37

In radioligand binding experiments the affinity (pK_i values) of UH-AH 37 for cortical M_1, cardiac M_2 and glandular M_3 binding sites were 8.05, 7 05 and 7.46, respectively. A comparison with pirenzepine showed that the affinities of both compounds for the M_1 receptor were similar However, the M_1 selectivity of UH-AH 37 was less pronounced. A striking difference
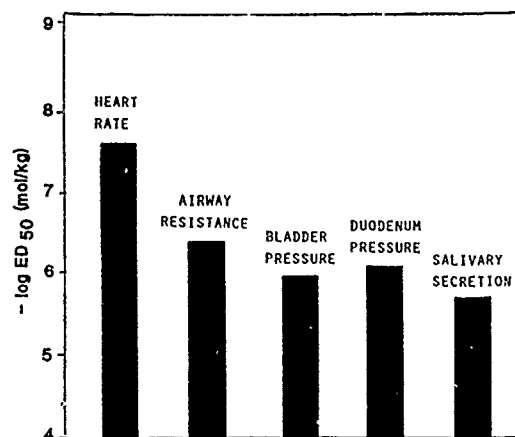

Fig 8 Potency of AQ-RA 741 in antagonizing the effects of methacholine (50 μg i v. or i a) in the anaesthetized cat

between UH-AH 37 and pirenzepine is the approximately tenfold higher affinity for ileal than for atrial muscarinic receptors[12].

The corresponding pA_2 values are 8.23 and 7.09, respectively (see Fig. 7). The pA_2 value for methacholine induced contraction of guinea-pig tracheal smooth muscle is 7.93. Recently, UH-AH 37 has been separated into its enantiomers[13]. It has been shown that the (+) enantiomer (UH-AH 371) is approximately 50–100 times more active than the (–) enantiomer. The selectivity profile of the active (+) enantiomer is similar to that of UH-AH 37. It should be stated that the selectivity profile of UH-AH 371 does not fit into the concept that M_3 receptors are the functional receptors responsible for contraction of ileal smooth muscle[12]. The selectivity profile of UH-AH 37 (racemate) and its active enantiomer is $M_{sm} \approx M_1 > M_3$, M_2.

Compounds that possess a high affinity for M_1 receptors as well as for those present in smooth muscle could be of use in the treatment of chronic obstructive lung disease. There are strong indications that M_1 receptors in the airways are involved in the regulation of airway smooth muscle tone[14]. Therefore, M_1- and

TABLE I Potency and selectivity of AF-DX 116, AF-DX 384 and AQ-RA 741 for muscarinic receptors *in vitro* and *in vivo*

Compound	In vitro					In vivo		
	receptor binding pK_i			functional pA_2		–log ED50 (mol/kg)		
	cortex (M$_1$)	heart (M$_2$)	gland (M$_3$)	heart	ileum	sympathetic ganglia[a] (M$_1$)	heart[b] (M$_2$)	salivary glands[c] (M$_3$)
AF-DX 116	6 3	7 1	5 7	6 9	6 2	5 6	6 4	5.0
AF-DX 384	7 5	8.2	7 0	8 6	7 6	5 9	7 4	6.0
AQ-RA 741	7 7	8.3	6 9	6 5	6 6	5 6	7 2	<5 0

[a] Inhibition of McN-A-343-induced pressor response in pithed rat, [b] inhibition of bradycardia due to vagal stimulation in pithed rat, [c] inhibition of pilocarpine-induced salivation in anaesthetized rat.

M_{sm}- selective compounds may be potent and selective bronchospasmolytics. To test this hypothesis we carried out functional studies in anaesthetized guinea-pigs and rabbits.

In the guinea-pig acetylcholine was administered (50 µg i.v. and i.a.) to evoke bradycardia and an increase in airway resistance. Approximately tenfold lower doses of UH-AH 371 were required to inhibit the agonist response of the airways (ED50 = 54 µg/kg i.v.) than that on heart rate. Additionally, experiments were performed in rabbits, where bradycardia and bronchoconstriction were elicited by stimulation of the right vagus. The ED50 of UH-AH 371 in antagonizing the vagally induced increase in airway resistance was 7.0 µg/kg, whereas the ED50 for the bradycardic response was inhibited with 466 µg/kg. Therefore, UH-AH 371 showed a striking bronchospasmolytic selectivity.

Conclusions

Tricyclic compounds play an important role in the identification and subclassification of muscarinic receptors. The real breakthrough leading to general acceptance of muscarinic receptor heterogeneity was accomplished with the M_1-selective drug pirenzepine. This concept of muscarinic receptor subtypes was further strengthened by the discovery of the M_2-selective compound AF-DX 116, an antagonist that provided clear-cut evidence for differences between cardiac and glandular muscarinic receptors. In subsequent structure–activity studies an analog of AF-DX 116, AQ-RA 741 was found which proved to be one of the most potent M_2 antagonists known so far. Moreover, its selectivity profile is comparable to that of methoctramine.

Recently, a novel M_1-selective compound UH-AH 37 has been discovered which showed differences from pirenzepine in functional studies. UH-AH 37 exhibits significantly higher affinity for ileal than for atrial muscarinic receptors.

On the basis of our present knowledge, we think that receptor selectivity of tricyclic muscarinic antagonists is strongly related to stereochemical features. Analyses of structure–activity and structure–selectivity relationships of a large number of pirenzepine and AF-DX 116 analogs suggest that it is the nature of the side-chain and the spatial placement of the basic nitrogen atom in relation to the tricyclic backbone that determine the differences in receptor subtype recognition. This hypothesis however has to be further substantiated

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Polymethylene tetraamines as muscarinic receptor probes

Carlo Melchiorre, Anna Minarini, Piero Angeli*, Dario Giardinà*, Ugo Gulini* and Wilma Quaglia*

The possibility that polymethylene tetraamines act as divalent ligands has been explored. Structure–activity relationship studies among polymethylene tetraamines have shown that four nitrogens are necessary for high affinity binding to M_2 receptors while being less important for M_3 muscarinic receptors. Replacement of one terminal methoxybenzyl group of the potent and selective muscarinic antagonist methoctramine by different moieties led to weaker antagonists suggesting that the two terminal nitrogens of methoctramine interact with two similar receptor sites Data are presented which suggest that methoctramine might interact with four acidic residues of the receptor: two residues are buried in the third transmembrane segment whereas the others are located extracellularly on the loop 4–5 which may represent the allosteric site where several antagonists such as gallamine bind. An hypothetical model describing the interaction of methoctramine with the M_2 receptor is proposed. It may provide a useful working hypothesis for the design of new selective muscarinic ligands.

The existence of multiple muscarinic receptors has been established by functional, binding and molecular biological studies[1]. At present, muscarinic receptors are classified into at least three pharmacologically well defined subtypes[2]: M_1, M_2 and M_3 (see p. VII). M_1 receptors have high affinity for pirenzepine[3] and are mainly located in the central nervous system and peripheral ganglia. M_2 receptors are found in cardiac cells and are characterized by a high affinity toward methoctramine[4,5], AF-DX 116[6], gallamine[7], and himbacine[8]. M_3 receptors are those in smooth muscles and exocrine glands and which display a high affinity for 4-DAMP[9], hexahydro-sila-difenidol and related compounds[10,11]. Both M_2 and M_3 muscarinic receptor types show low affinity to pirenzepine[12].

Recent cloning studies have confirmed the heterogeneity of muscarinic receptors from different species[13-17]. Five different muscarinic receptor proteins (m1–m5) have been cloned which means that two receptor types (m4 and m5) are not characterized yet from a pharmacological point of view. Despite the impressive results obtained by molecular biology studies, much remains to be learned of the relationship between the acetylcholine binding site and the functional machinery of muscarinic receptors, and only little is known of the topography of the acetylcholine or antagonist binding sites. The latter relationship assumes increased importance if one considers that agonists and antagonists may occupy different binding sites[18,19]. As part of our attempts to characterize muscarinic receptor subtypes and to elucidate the mode of binding of antagonists, we have developed a series of potent and selective muscarinic antagonists (the prototype of which is methoctramine, Fig. 1). Here we summarize an approach we have employed in the design of methoctramine-related antimuscarinic

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ligands in order to verify whether polymethylene tetraamines act as divalent ligands interacting with two vicinal binding sites

Divalent ligand approach

The possibility that polymethylene tetraamines behave as divalent ligands, which can be defined as molecules that incorporate two discrete pharmacophores linked through a spacer, derives from their evident symmetry (Fig. 1). This may indicate that the two 1,ω-alkanediamine moieties (pharmacophore a) or simply the two terminal N-substituted groups (pharmacophore b) of methoctramine (Fig. 1: $n = 6$, $m = 8$) could recognize two similar if not identical sites. The length of the spacer could be responsible for the selectivity by allowing bridging in one receptor subtype, but not in another, as schematically shown in Fig. 2. In the light of this it is noteworthy that polyvalent ligands have been of value in probing the interbinding site distances at pharmacological receptors A classical example of such ligands is represented

Pharmacophore b

Pharmacophore a

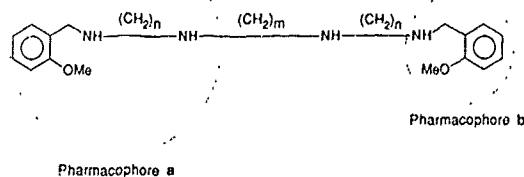*Fig 1 Chemical structure of polymethylene tetraamines, the prototype of which is methoctramine ($n = 6$ $m = 8$) Circles indicate that 6-(2-methoxy)benzyl-1,6-hexanediamine (a) and 2-methoxybenzylamine (b) are possible pharmacophores of tetraamines considered as divalent ligands In a divalent ligand two molecules of a pharmacophore are linked via a spanning chain*
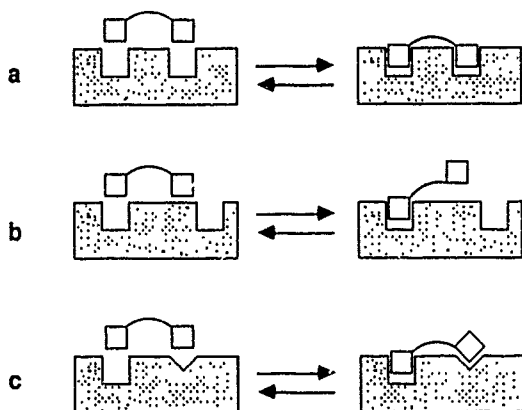
Fig 2 Possible mechanisms of interaction of a divalent ligand with a pharmacological receptor The squares represent the pharmacophore whereas the shaded areas indicate the receptor with its binding sites (a) The length of the spacer permits the divalent ligand to occupy two vicinal sites which are identical or homologous (b) The length of the spacer does not allow the divalent ligand to bridge two sites, only monovalent binding will occur (c) Provided that spacer length allows interaction, the divalent ligand may occupy a recognition receptor site and, with the second pharmacophore, a neighbouring accessory site which is unique for that receptor

by the bis-onium cholinergic blocking agents[20]. More recently, the divalent ligand approach has been used successfully to probe opiate receptor subtypes[21-24].

The selectivity of a divalent ligand can, in principle, be ascribed to two mechanisms[24]. A first mode of interaction is possible when the length of the spacer is long enough to allow both pharmacophores to occupy two similar if not identical vicinal receptor sites (Fig. 2a) whereas the other possible mechanism involves the bridging of a receptor site and a neighboring accessory site (Fig. 2c). Although the additional binding energy to an accessory site might be lower than that to a recognition receptor site, it can, nevertheless, contribute to the selectivity of a divalent ligand.

Design strategy

Polymethylene tetraamines were first developed as α-adrenoreceptor antagonists (the prototype of which is benextramine)[25]. However, the significant antimuscarinic activity displayed by benextramine stimulated our interest in defining the structural requirements which would have enhanced antimuscarinic potency while having no or low affinity for α-adrenoreceptors. It oon became apparent that the disulfide bridge of benextramine is of paramount importance for α-adrenoreceptor inhibition whereas it is absolutely unnecessary for antimuscarinic potency since its replacement by two methylenes yielded a compound which retained affinity for muscarinic receptors but lost α-adrenoreceptor blocking properties[26,27]. Thus, polymethylene tetraamines lacking the disulfide moiety were the starting point in the design of new antimuscarinic agents. Three types of structural modifications were performed on benextramine in order to determine

which features contribute to affinity and whether some of these factors could be manipulated to create selectivity at muscarinic receptor subtypes[28,29]:

1. Variation in the length of the carbon chain separating inner from outer nitrogens as well as inner nitrogens;
2. Incorporation of substituents on the four nitrogens;
3 Variation in the number of basic nitrogens.

Structure–activity relationships

The carbon chain length separating the four nitrogens of polymethylene tetraamines proved to play a crucial role in both affinity and selectivity toward M_2 but not M_3 muscarinic receptor subtypes (Fig. 3). It is evident that all tetraamines studied were at least one order of magnitude more potent in the atrium than in ileum, which clearly indicates that the carbon chain length has a divergent effect at M_2 and M_3 muscarinic receptors. The result was that potency in the atrium (M_2) has a definite peak for methoctramine, which bears an eight-carbon chain (m) between the inner nitrogens and a six-carbon chain (n) separating the inner from the outer nitrogens whereas the affinities of methoctramine and of its homologs in the ileum (M_3) not only were lower than that in the atrium but also did not differ significantly from each other The conclusion is that optimum affinity at M_2 receptors depends on the number of methylenes whereas at M_3 receptors the antimuscarinic potency of tetraamines is not related to that distance.

In attempting to answer the question of whether a tetraamine moiety or a 1,ω–alkanediamine or simply a N,N-disubstituted amine might be the pharmacophore,
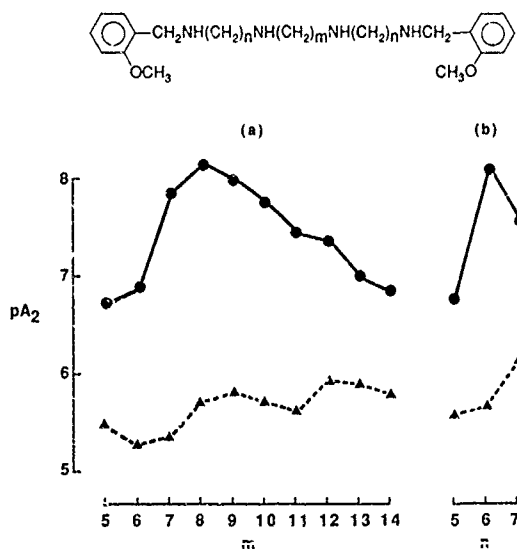

Fig. 3. Relationship between muscarinic antagonist potency and the carbon chain length in polymethylene tetraamines in guinea-pig paced left atrium (●) and guinea-pig and rat ileum (▲), (a). n = 6, m = 5–14, (b) n = 5–7, m = 8 (Data taken from Refs 28 and 29)

TABLE I Affinity constants for unsymmetrical amines (incorporating one, two or three fewer nitrogens than methoctramine) for muscarinic receptors in guinea-pig left atrium (M_2) and ileum (M_3)
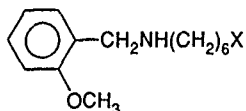
X	pA$_2$		M$_2$: M$_3$ selectivity ratio
	atrium	ileum	
Methoctramine	7 97 ± 0 05	5 92 ± 0 04	112
-H HCl	5 12 ± 0.05^a	<5^b	>1
-NH$_2$ 2HCl	5 14 ± 0 09^a	5 0 ± 0 14^a	1
-NHCH$_3$ 2HCl	5 40 ± 0 05^a	<5^b	>3
-NH(CH$_2$)$_3$CH$_3$ 2HCl	5 48 ± 0 06	<5^b	>3
-NH(CH$_2$)$_8$NH$_2$ 3HCl	6 16 ± 0.04	5 28 ± 0 06	8
-NH(CH$_2$)$_8$NHCH$_3$ 3HCl	6 70 ± 0 06	5 83 ± 0 04	7

Experiments were performed as described in Ref 29 pA$_2$ values ± s e were calculated from Schild plots, constrained to slope −1 0 It was always verified that the experimental data generated a line whose derived slope was not significantly different from unity ($p > 0.05$) Dose–ratio values were calculated at three different antagonist concentrations, and each concentration was tested at least five times Parallelism of dose–response curves was checked by linear regression, and the slopes were tested for significance ($p < 0 05$) The M$_2$ M$_3$ selectivity ratio is the antilog of the difference between the pA$_2$ values at atrial and ileal muscarinic receptors, respectively Carbachol was the agonist used
^{a} pK_b value derived from a single concentration ^{b} Inactive up to a concentration of 10 μM

it became essential to verify the importance of the four basic nitrogens on activity among the polymethylene tetraamine class of antimuscarinic agents. Unsymmetrical analogues of methoctramine incorporating one, two or three fewer nitrogens were therefore synthesized and, as can be seen in Table I, sharp decreases in M$_2$ muscarinic blocking activity were observed after removal of either one or two nitrogens. The monoamine (X = H; Table I) is as active as diamines [X = NH$_2$, NHCH$_3$, NH(CH$_2$)$_3$CH$_3$; Table I], which indicates that the second nitrogen apparently does not contribute significantly either to affinity or to selectivity. However, the most striking result was the finding that monoamine and diamines are not only almost three orders of magnitude less potent than methoctramine at M$_2$ receptors but also incapable of discriminating between M$_2$ and M$_3$ muscarinic receptor types. The triamines [X = NH(CH$_2$)$_8$NH$_2$, NH(CH$_2$)$_8$NHCH$_3$; Table I] are significantly more potent at M$_2$ than at M$_3$ receptors although to a lesser extent than methoctramine.

In order to investigate further the role of basic nitrogens, we replaced alternatively inner and outer nitrogens with ether and/or amide functions. The results assembled in Table II clearly indicate that four amine functions are required for optimum activity at M$_2$ muscarinic receptors.

Finally, in order to define the structural requirements of the binding sites where the two terminal nitrogens of methoctramine interact, it was necessary to prepare unsymmetrical tetraamines in which one of the two 2-methoxybenzyl groups was replaced by other moieties. Table III clearly shows that optimum activity at M$_2$ muscarinic receptors is retained by methoctramine which bears a 2-methoxybenzyl group on both terminal nitrogens. In fact, replacement of even one 2-methoxy function by a hydrogen caused a definite, albeit modest decrease in potency. Replacement of the second 2-methoxy function by another hydrogen atom yielding a symmetrical tetraamine, the benzyl analogue of methoctramine (not shown)[29], resulted in a further decrease in M$_2$ muscarinic blocking activity. A similar effect was observed by replacing the 2-methoxybenzyl group with a methyl or a hydrogen (Table III). These results indicate that the binding sites, where the terminal nitrogens of methoctramine interact, may have similar if not identical structural requirements.

Mode and site of action

The results obtained so far with methoctramine clearly indicate that we are faced with the most selective M$_2$ muscarinic antagonist available today and point to a dual mode of interaction with the M$_2$ muscarinic receptor (for reviews, see Refs 4, 5 and 30): competitive at low concentration (≤ 1 μM) and allosteric at high concentrations (> 1 μM). This derives from the observation that, in contrast with functional experiments in paced left atria where a competitive mechanism of inhibition of M$_2$ receptors by methoctramine has always been found[31], high concentrations (> 1 μM) of methoctramine markedly influenced the dissociation of [^{3}H]NMS[32] and [^{3}H]dexetimide[33] from cardiac membranes, in agreement with a noncompetitive mode of action. It should be noted, however, that methoctramine displays such noncompetitive behavior at a concentration 100-fold higher than its affinity constant at M$_2$ receptors. Very recently, another cardioselective antagonist, AF-DX 116, has been shown to exhibit allosteric

TABLE II Affinity values for diamines related to methoctramine for muscarinic receptors in guinea-pig left atrium (M$_2$) and ileum (M$_3$)
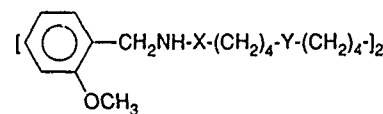
X	Y	pA$_2$		M$_2$: M$_3$ selectivity ratio
		atrium	ileum	
CH$_2$	CH$_2$NH^a	7 97 ± 0 05	5 92 ± 0 04	112
CH$_2$	CONH^b	6 30 ± 0.10	5.35 ± 0 14^c	9
CO	CH$_2$NH^b	6 57 ± 0.02	6 52 ± 0.15	1
CH$_2$	CH$_2$O^d	<5^e	<5^e	—

For experimental details, see legend to Table I.
^{a} Methoctramine ^{b} Obtained as dioxalate salt ^{c} pK_b value derived from a single concentration ^{d} Obtained as dihydrochloride salt. ^{e} Inactive up to a concentration of 10 μM.

TABLE III Affinity values for unsymmetrical tetraamines related to methoctramine for muscarinic receptors in guinea-pig left atrium (M_2) and ileum (M_3)
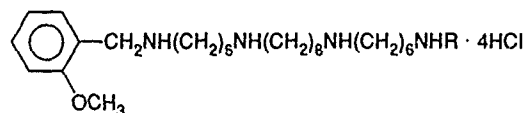
$$\langle\bigcirc\rangle-CH_2NH(CH_2)_6NH(CH_2)_8NH(CH_2)_6NHR \cdot 4HCl$$
$$|$$
$$OCH_3$$

R	pA₂		$M_2:M_3$ selectivity ratio
	atrium	ileum	
2-CH₃O-benzyl[a]	7 97 ± 0 05	5 92 ± 0.04	112
Benzyl	7 56 ± 0 03	5 82 ± 0 05	55
CH₃	7 37 ± 0.07	5.62 ± 0 08	56
H	7.08 ± 0 06	5 71 ± 0 06	23

For experimental details, see legend to Table I
[a] Methoctramine.

antagonism, behaving like gallamine and methoctramine in slowing the dissociation of [³H]dexetimide from cardiac muscarinic receptors[33]. These findings are not surprising if one considers that the cardiac muscarinic receptor bears an allosteric site at which several compounds bind. This has been observed with gallamine[7], hemicholinium[34], alkane-bis-ammonium compounds[35,36], Ca^{2+} antagonists[37] and antiarrhythmics[38]

Whatever the interaction mechanism of methoctramine may be at high concentrations, it has been demonstrated that in the range 10–1000 nM it is a competitive antagonist in both functional and binding studies[4,5]. Furthermore, the high affinity of methoctramine in binding to rat and bovine cardiac membranes parallels the affinity found in functional assays with cardiac preparations[5]. Moreover, binding results correlate with functional *in-vivo* studies where methoctramine discriminates markedly between cardiac M_2 and ganglionic M_1 muscarinic receptors in the pithed rat[32,39].

A second very interesting aspect concerning polymethylene tetraamines relates to their possible site of action. The finding that four basic nitrogens are required for optimum activity (Tables I and II) strongly suggests that methoctramine interacts with four nucleophiles located on the receptor. Furthermore, the observation that in the symmetrical polymethylene tetraamines, where the bridging carbon chain length between two molecules of the monovalent ligand 6-(2-methoxy)benzyl-1,6-hexanediamine was changed from 5 to 14 methylenes (Fig. 3), affinity for M_2 receptors varied significantly with the spacer length and a definite peak of activity was observed at 8 methylenes clearly suggests that tetraamines may interact with two sites at an appropriate distance from each other. Most mechanistic formulations assume that the primary event in drug–muscarinic receptor interaction involves the cationic head group of the drug (the quaternary ammonium or protonated amine moiety) and an anionic group on the receptor presumably

provided by an aspartate or a glutamate residue. An analysis of the primary structure of cloned muscarinic receptors[13-17] reveals that only five acidic residues are conserved among the amino acid sequences of the different receptors, aspartates 69, 97, 103 and 120 and glutamate 382 (porcine m2 sequence numbering)[11]. Since glutamate 382 is intracellularly located, the four aspartate residues are prime candidates for the site of interaction by muscarinic ligands, both agonists and antagonists. Curtis *et al.*[18] and Hulme *et al* [40] reported recently that [³H]propylbenzylcholine mustard alkylates two carboxylate groups, which were shown by the peptide mapping technique to reside within the third transmembrane segment. On the basis of site-directed mutagenesis studies, the hypothesis was advanced that aspartate 113 of the β-adrenergic receptor (conserved as aspartate 103 in m2 receptors) is part of the ligand binding site[19,41]. Considering the analogy

Cytoplasm
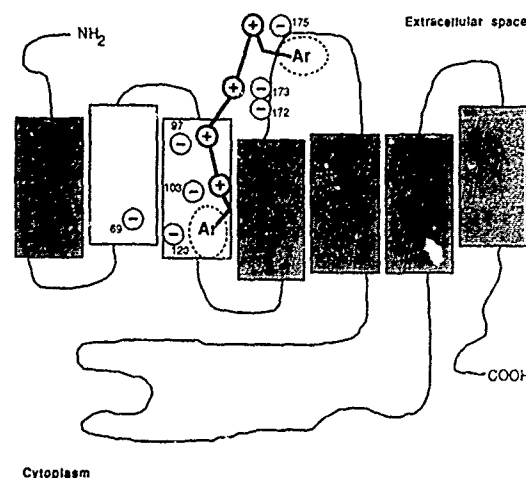
Fig 4 Schematic representation of the interaction between polymethylene tetraamines and M_2 muscarinic receptors The receptor is illustrated with its seven transmembrane segments (shaded rectangles) connected to each other by intra- (1–2, 3–4 and 5–6) and extracellular (2–3, 4–5 and 6–7) loops (solid lines) The acidic residues, aspartates 69 97, 103 and 120 in transmembrane domains II and III, and aspartate 173 and glutamates 172 and 175 in the extracellular loop 4–5, are indicated by ⊖ to denote their anionic nature Methoctramine is represented with a bold line connecting four positive charges (the four basic nitrogens) and ending with Ar (2-methoxybenzyl groups) The primary event of the interaction might take place between a terminal nitrogen of methoctramine and glutamate 175 and aspartate 173 (or glutamate 172) on the extracellular loop 4–5 This binding would be reinforced by the interaction of Ar with an appropriate area (broken circle) and would cause by way of a conformational change, the penetration of methoctramine into the third transmembrane domain of the receptor followed by the interaction with aspartates 97 and 103 This hypothetical mode of action does not apply to other muscarinic receptor subtypes because aspartate 173 and glutamates 172 and 175 are not conserved thus giving a possible explanation for the selective binding of methoctramine

between β-adrenergic and muscarinic receptors, a similar role for the corresponding aspartate of muscarinic receptors in binding the positively charged amine group of the muscarinic ligands has been proposed[41]. Thus, it appears that several aspartate residues might be involved in binding muscarinic ligands.

Although data on the protonation of the four nitrogens of methoctramine in physiological salt solution are not available yet, we have proposed[30] that the four positively charged nitrogens of methoctramine interact with the four aspartate residues located in the second and third transmembrane segments. The importance of positively charged nitrogens of methoctramine has been confirmed by the low activity shown by compounds obtained by replacing an amine function with an amide or an ether moiety (Table II). Although in agreement with the previously proposed topographical model[30], present results can be rationalized by admitting that methoctramine may interact differently with the receptor. In fact, the amino acid sequence of m2 muscarinic receptors incorporates three acidic residues in the loop 4–5, aspartate 173 and glutamates 172 and 175, which are not conserved in other muscarinic receptors and which may account for selective binding of certain antagonists such as methoctramine if one considers that these residues are easily accessible since they are located in the extracellular space. The results assembled in Table I support this view since diamines [X = NH_2, $NHCH_3$ and $NH(CH_2)_3CH_3$], that is, half methoctramine, are weak antagonists at both M_2 and M_3 receptors, suggesting that the high affinity of tetraamines for M_2 receptors is the result of an additional interaction at a second site, which is lacking in M_3 receptors. Figure 4 shows an hypothetical model in which methoctramine interacts with aspartates 97 and 103 located in the third transmembrane segment. A further interaction with aspartate 173 or glutamate 172 and glutamate 175 located in the loop 4–5 may represent its interaction with the allosteric site where antagonists such as gallamine also bind. Thus, in this model, aspartates 69 and 120, which are buried into the membrane, are possible but less likely contenders for the binding of methoctramine than the acidic residues located in the loop 4–5. This view is in agreement with the peptide mapping studies and the general belief that the binding site, which may be represented by aspartate 103 in analogy with the β-adrenergic receptor[19,41], has to be at least partly exposed to the extracellular side.

Acknowledgements

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Pharmacology of hexahydro-difenidol, hexahydro-sila-difenidol and related selective muscarinic antagonists

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A series of hexahydro-difenidol (HHD) and hexahydro-sila-difenidol (HHSiD) analogues modified in the amino group, the phenyl ring and in the alkylene chain were investigated for their binding and functional properties at muscarinic M_1, M_2 and M_3 receptors. Novel muscarinic receptor antagonists were obtained which exhibited different receptor selectivity profiles from the parent compounds HHD and HHSiD ($M_1 = M_3 > M_2$), e.g. HHD and HHSiD methiodides, $M_1 > M_2 = M_3$; p-fluoro-HHSiD, $M_3 > M_1 > M_2$; trans-hexbutinol, $M_1 > M_3 > M_2$; and (s)-p-fluoro-hexbutinol, $M_3 > M_2 = M_1$. Stereoselectivity ratios [(R)/(s)] for the enantiomers of HHD, hexbutinol and p-fluoro-hexbutinol were highest at M_1, intermediate at M_3 and lowest at M_2 receptors.

A series of structurally related carbon/silicon analogues of the antiparkinsonian drug pridinol were investigated for their binding and functional properties at muscarinic receptor subtypes (Figs 1 and 2)[1-6]. The binding affinities of the compounds were similar to their antimuscarinic potencies in functional experiments. All compounds with a centre of chirality were investigated in these studies as racemates.

Pridinol exhibited high affinity for M_1 receptors in NB-OK 1 cells whereas its affinities for M_2 receptors in rat heart and M_3 receptors in rat striatum were lower by factors of 10 and 2.5, respectively (Fig. 1). Trihexyphenidyl, procyclidine and biperiden showed qualitatively similar selectivity profiles to that of pridinol ($M_1 > M_3 > M_2$), but the affinities of trihexyphenidyl and biperiden for M_1 over M_2 receptors were higher (25-fold) than those of pridinol and procyclidine (10-fold). Replacement of the central carbon atom by a silicon atom in these compounds had only moderate influence on affinity and selectivity. In contrast, the pyrrolidino analogue pyrrinol was a weaker antimuscarinic agent with a small preference for M_1 and M_3 over M_2 receptors (= 3.5-fold). Sila-substitution of pyrrinol increased its affinity for all subtypes up to

13-fold, the increase being greatest for M_1 receptors. Thus, sila-pyrrinol showed the same receptor selectivity profile as pridinol.

Altering the chain length between the amino group and the central atom 'El' from two to three methylene groups in trihexyphenidyl, procyclidine and their silicon analogues (Fig. 1) changed the receptor selectivity profile of these compounds. Thus, HHD, homoprocyclidine and their silicon analogues exhibited similar high affinities for M_1 receptors in rabbit vas deferens and for M_3 receptors in guinea-pig ileum, whereas their affinity for M_2 receptors in guinea-pig atria was much lower (Fig. 2). Like pyrrinol (Fig. 1), difenidol and homo-pyrrinol were weaker antagonists than their silicon analogues (Fig. 2). However, the increase in antimuscarinic potency produced by sila-substitution again was not the same for all receptor subtypes. Thus, sila-difenidol and homo-sila-pyrrinol showed selectivity profiles different from their carbon analogues and gave a better differentiation between M_2 and M_3 receptors.

Hexahydro-(sila-)difenidol analogues

Structural modifications of HHD and HHSiD molecules were undertaken in a search for M_3-selective antagonists. Changes included introduction of substituents in the phenyl ring, N-methylation of the piperidino ring and incorporation of a double or a triple bond into the alkylene chain (Fig. 3). Although, in both functional and binding studies, the racemic parent compounds HHD and HHSiD showed the same receptor selectivity profile ($M_1 = M_3 > M_2$) (Table I), structural modifications resulted in differences in these profiles. As all these analogues possess a centre of chirality, the affinities of the individual enantiomers of HHD, hexbutinol and p-fluoro-hexbutinol were also investigated.

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Fig 1 Affinity values (pK_i) of antagonists (C/Si pairs) in inhibiting specific [3H]NMS binding at muscarinic M_1 receptors in human NB-OK 1 cells (■), M_2 receptors in rat heart (●) and M_3 receptors in rat pancreas [(sila)trihexyphenidyl, (sila-)procyclidine, (sila)biperiden] or rat striatum [(sila-)pridinol, (sila)pyrrinol] (▲) Experiments were performed as previously described[1,14]. Competition curves were analysed by fitting the data to a one binding site model
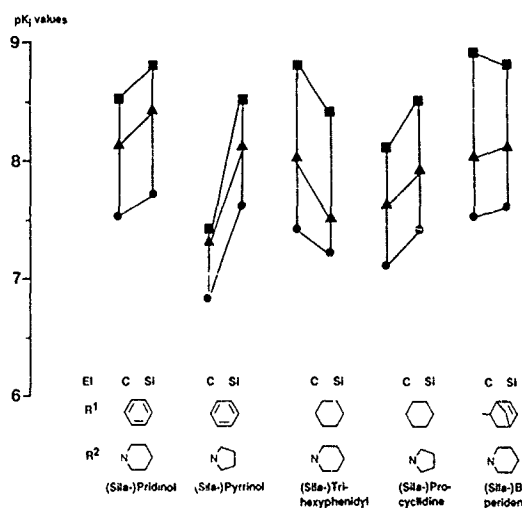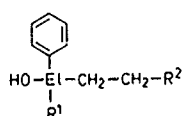
Effect of substituents in the phenyl ring

A methoxy substituent in the *ortho*, *meta* or *para* position or a *p*-chloro substituent in the phenyl ring of HHSiD reduced the affinity to the three muscarinic receptor subtypes up to 20-fold. The *o*-methoxy and *m*-methoxy analogues retained qualitatively the same selectivity profile as the parent compound HHSiD ($M_1 \simeq M_3 > M_2$), but the *p*-methoxy and *p*-chloro derivatives showed a small (2.5-fold) preference for M_3 receptors, which was more pronounced in the case of the *p*-fluoro analogue, *p*-fluoro-HHSiD (Table II). This selectivity ($M_3 > M_1 > M_2$) is due to the fact that the *p*-fluoro substituent led to a decrease in M_1 and M_2 antimuscarinic potency with minimal influence on the affinity for M_3 receptors compared to HHSiD.

In contrast, the affinity profile of the corresponding carbon analogue *p*-fluoro-HHD was similar to that of the parent compound HHD (Table I) The affinity values (pA_2) of *p*-fluoro-HHD in rabbit vas deferens (M_1), guinea-pig atria (M_2) and ileum (M_3) were 7.6, 6.6 and 7.9, respectively. Thus, while the affinity and selectivity of HHD and HHSiD were essentially similar (Table I), those of the two *p*-fluoro derivatives were different.

The affinities of *p*-fluoro-HHSiD were also determined using ganglionic (M_1), atrial (M_2) and ileal (M_3)

Fig 2 Antimuscarinic potencies (pA_2 values) of C/Si pairs at muscarinic M_1 receptors in rabbit vas deferens (■), M_2 receptors in guinea-pig atria (●) and M_3 receptors in guinea-pig ileum (▲) Experiments were performed as previously described[1–3]
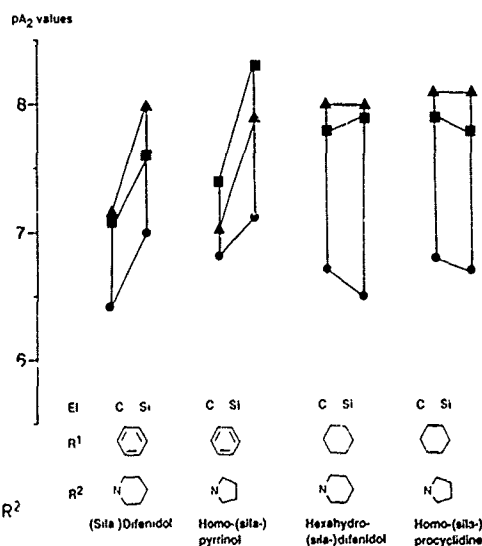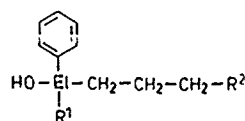
Fig 3. Structural modifications of hexahydro-difenidol (El = C) and hexahydro-sila-difenidol (El = Si). (*, chiral centre.)

preparations of the rat[3] and in binding studies using homogenates of calf superior cervical ganglia (M_1). As shown in Table III, the affinity values of p-fluoro-HHSiD in calf ganglia and rat atria and ileum were similar to those found in rabbit vas deferens and guinea-pig atria and ileum, respectively (Table II). Although the pA_2 value at M_1 receptors in rat supe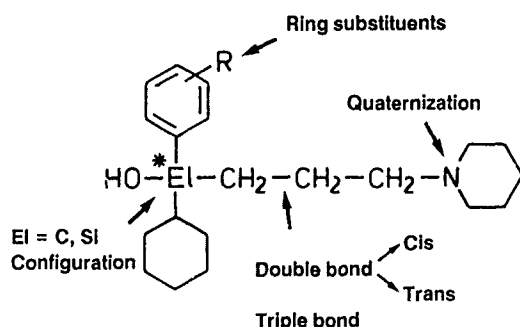rior cervical ganglia was about threefold higher than that found in rabbit vas deferens, overall these data suggest that the receptor selectivity profile of p-fluoro-HHSiD and that of the parent compound HHSiD are species independent[3].

Effect of N-methylation

HHD and HHSiD methiodides were investigated for their binding affinities in NB-OK 1 cells (M_1), rat heart (M_2) and pancreas (M_3) (Table I)[1]. Quaternization of HHD and HHSiD increased the affinity for M_1 and M_2 receptors up to 20-fold, this increase being greatest at cardiac M_2 receptors. In contrast, the affinity of HHD for M_3 receptors in pancreas was not changed by N-methylation, and that of HHSiD was increased only 2.5-fold. Thus, N-methylation of the tertiary amines changed the receptor selectivity pattern from $M_1 \approx M_3 > M_2$ to $M_1 > M_3 \approx M_2$ for the two methiodides.

TABLE I. Affinity values from binding (pK_i) and functional (pA_2) studies of hexahydro-difenidol (HHD), hexahydro-sila-difenidol (HHSiD) and their methiodides HHD+ and HHSiD+ at muscarinic receptor subtypes

Drug	M_1		M_2		M_3	
	pK_i	pA_2	pK_i	pA_2	pK_i	pA_2
HHD	7.9	7.8	6.8	6 7	7.9	8 0
HHSiD	7.9	7 9	6 8	6.5	7 8	8 0
HHD+a	8 6		8.1		7 9	
HHSiD+a	8 8		8 0		8 2	

Binding experiments were performed in NB-OK 1 cells (M_1), rat heart (M_2) and pancreas (M_3) using [3H]NMS as radioligand[1] pA_2 values were determined using rabbit vas deferens (M_1), guinea-pig atria (M_2) and ileum (M_3)[1–3,12]. apA_2 not determined.

Similar results were obtained with p-fluoro-HHSiD and its methiodide (Table II). N-methylation of the tertiary amine increased the affinity for M_1 receptors in rabbit vas deferens and M_2 receptors in guinea-pig atria by 25- and 50-fold, respectively, whereas the affinity for M_3 receptors in guinea-pig ileum was not affected, thus abolishing the selectivity between M_2 and M_2 receptors.

Olefinic analogues of hexahydro-difenidol

HHD is a flexible molecule and little is known about the conformation involved in its interaction with muscarinic receptor subtypes[7]. Therefore, conformationally restricted olefinic analogues of HHD may be of value in investigating the stereochemical requirements for blockade of muscarinic receptor subtypes[8]. Introduction of a double bond into the HHD molecule (Fig. 4) with substituents in trans configuration (trans-hexbutenol) increased the affinity for M_1 and M_2 receptors by factors of 10 and 6.3, respectively, whereas the

TABLE II pA_2 values of hexahydro-sila-difenidol (HHSiD) and phenyl ring-substituted analogues for muscarinic receptors

Drug	pA₂ values		
	M_1	M_2	M_3
HHSiD	7 9	6 5	8.0
o-CH₃O-HHSiD	6 8	5 8	6 7
m-CH₃O-HHSiD	6 7	5 5	6 7
p-CH₃O-HHSiD	6 7	5 6	7 1
p-Cl-HHSiD	6 7	5.5	7 1
p-F-HHSiD	6.7	6 0	7 8
p-F-HHSiD methiodide	8 1	7 7	7 8

Values were determined as indicated in Table I[2,3,12]

affinity for M_3 receptors was not significantly char ~d (Table IV). Thus, trans-hexbutenol showed a receptor selectivity profile ($M_1 > M_3 > M_2$) different from that of HHD The affinity of cis-hexbutenol was lower than that of the trans-isomer for the three subtypes, indicating that extended rather than folded conformations are more important for the interaction of HHD with the muscarinic receptors.

The difference in affinity of cis- and trans-hexbutenol was highest at M_1 (50-fold), intermediate at M_2 (tenfold) and lowest at M_3 receptors (fourfold), resulting in different receptor selectivity profiles for the two isomers (cis-hexbutenol, $M_3 > M_1 > M_2$; trans-hexbutenol, $M_1 > M_3 > M_2$).

Acetylenic analogues of hexahydro-difenidol and effect of absolute configuration

Acetylenic analogues of HHD, the carbon chain of which is constrained to be linear, were also investigated (Table V; Fig. 5).

(R)-HHD exhibited high affinity for M_1 receptors in rabbit vas deferens as well as for M_3 receptors in guinea-pig ileum. Its affinity for M_2 receptors in guinea-pig atria was lower by factors of 50 and 25,

TABLE III. pK_i and pA_2 values of hexahydro-sila-difenidol (HHSiD) and p-fluoro-hexahydro-sila-difenidol (p-F-HHSiD) at muscarinic receptor subtypes

Drug	Calf superior cervical ganglia[a] pK_i (M$_1$)	Rat superior cervical ganglia[b] pA_2 (M$_1$)	atria pA_2 (M$_2$)	ileum pA_2 (M$_3$)
HHSiD	7.6	7 5	6.2	7.8
p-F-HHSiD	6 9	7.2	6 0	7.9

[a]Binding experiments were performed with CSCG homogenates using [^{3}H](+)-telenzepine as radio-ligand[13]
[b]Antagonism of muscarine-induced depolarization[3].

respectively. Introduction of a triple bond into (R)-HHD increased the affinity for M$_2$ and M$_3$ receptors by factors of 6 and 2.5, respectively, whereas the affinity for M$_1$ receptors was not significantly changed. Thus, while (R)-hexbutinol shows a receptor selectivity profile qualitatively similar to that of (R)-HHD, the degree of selectivity for M$_1$ and M$_3$ over M$_2$ receptors is smaller than that of (R)-HHD (Table V). The high antimuscarinic potency of (R)-hexbutinol substantiates the conclusion that extended rather than folded conformations of HHD interact with the muscarinic receptors.

The affinity of (R)-hexbutinol for the three muscarinic receptors was reduced by p-fluoro substitution, but (R)-p-fluoro-hexbutinol showed a small preference for M$_3$ over M$_1$ receptors (2.5-fold), and the selectivity for M$_3$ over M$_2$ receptors was enhanced.

In general, the (s)-enantiomers were less potent than the (R)-isomers. However, introduction of a triple bond into the HHD molecule as well as p-fluoro substitution

Fig. 4 Chemical structure of cis-hexbutenol (top) and trans-hexbutenol (bottom).

of hexbutinol had different effects on affinity of the (s)-isomers in comparison with the (R)-enantiomers at the three subtypes. (s)-HHD was a very weak muscarinic antagonist showing no muscarinic receptor selectivity. Introduction of a triple bond into (s)-HHD increased the affinity for the three subtypes up to tenfold, but there was also no muscarinic receptor selectivity.

Comparison of (s)-hexbutinol and (s)-p-fluoro-hexbutinol showed that the p-fluoro derivative exhibited a higher affinity for M$_3$ receptors, whereas its antimuscarinic potency at M$_1$ and M$_2$ receptors was the same. Thus, fluoro substitution in the para position of the phenyl ring of (s)-hexbutinol enhanced its low M$_3$ selectivity. The receptor selectivity profile of (s)-p-fluoro-hexbutinol (M$_3$ > M$_2$ ≃ M$_1$) is different from that of the (R)-enantiomer (M$_3$ > M$_1$ > M$_2$) and p-fluoro-HHSiD (M$_3$ > M$_1$ > M$_2$) (Tables II and III).

As far as stereoselectivity is concerned, there are two noteworthy findings (Table V). First, stereoselectivity at the three muscarinic receptor subtypes is greatest for

TABLE IV Comparison of the affinities (pA_2 values) of hexahydro-difenidol and of cis- and trans-hexbutenol at muscarinic receptors

Drug	pA_2 values[a]			Stereoselectivity[b]		
	M$_1$	M$_2$	M$_3$	M$_1$	M$_2$	M$_3$
Hexahydro-difenidol	7.8	6 7	8 0			
trans-Hexbutenol	8.8	7 5	8.1	50	10	4
cis-Hexbutenol	7 1	6 5	7 5			

[a]pA_2 values were determined as indicated in Table I.
[b]trans/cis ratios, given as a measure of stereoselectivity, were calculated from the antilogs of the differences between respective pA_2 values.

HHD, intermediate for hexbutinol and lowest for p-fluoro-hexbutinol; this stereoselectivity is not generally associated with high affinity of the more potent isomer. Second, the stereoselectivity ratios consistently show the same order: M$_1$ > M$_3$ > M$_2$. This implies that the stereochemical requirements of the muscarinic receptor subtypes are different for the enantiomers of HHD, hexbutinol and p-fluoro-hexbutinol, being most stringent at M$_1$ receptors. Thus, stereoselectivity ratios can be used successfully as a parameter to characterize muscarinic receptor subtypes. Similar results have been obtained with the enantiomers of telenzepine[9], biperiden[10] and trihexyphenidyl[11].

Conclusions

The affinity and selectivity for muscarinic receptor subtypes of pridinol/sila-pridinol and HHD/HHSiD analogues are controlled by: the nature of the central atom 'El' (carbon or silicon) and the absolute configuration at 'El'; the nature of the ring systems bound to 'El';

TABLE V. pA_2 values and stereoselectivity [(R)/(S)] ratios of the enantiomers of hexahydro-difenidol (HHD), hexbutinol (HBN) and p-fluoro-hexbutinol (p-F-HBN) at muscarinic receptors

	pA_2 values[a]			Stereo-selectivity[b]		
Drug	M_1	M_2	M_3	M_1	M_2	M_3
(R)-HHD	8.7	7.0	8 4	501	16	200
(S)-HHD	6.0	5 8	6.1			
(R)-HBN	8.8	7 8	8.8	100	10	50
(S)-HBN	6.8	6.8	7.1			
(R)-p-F-HBN	8.1	7.0	8.5	32	1 6	8
(S)-p-F-HBN	6 6	6.8	7.6			

[a]pA_2 values were determined as indicated in Table I.
[b]These values were calculated from the antilogs of the differences between the respective pA_2 values

	R	A
HHD	H	CH_2CH_2
HBN	H	$C \equiv C$
p-F-HBN	F	$C \equiv C$
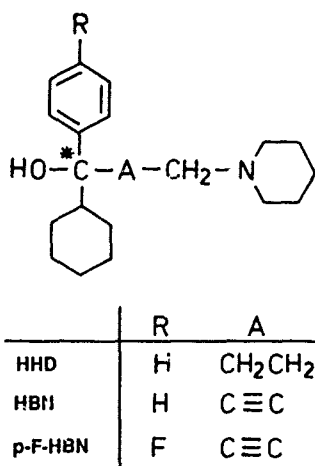
Fig. 5 Chemical structure of hexahydro-difenidol (HHD), hexbutinol (HBN) and p-fluoro-hexbutinol (p-F-HBN) The asterisk denotes the centre of chirality.

the structure of the amino (ammonium) group; the nature (length, presence of multiple bonds) of the carbon chain that connects 'El' and the amino group; substituents on the phenyl ring.

The structural and spatial requirements for binding antagonists are different for M_1, M_2 and M_3 muscarinic receptors, resulting in different receptor selectivity profiles for the pridinol and HHD derivatives. It is interesting that p-fluoro-HHSiD and (s)-p-fluoro-hexbutinol are M_3-selective antagonists. The data suggest that muscarinic receptor subtypes may be identified using stereoselectivity ratios of enantiomers of chiral antagonists such as HHD, hexbutinol and p-fluoro-hexbutinol.

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Stereoselectivity of the interaction of muscarinic antagonists with their receptors

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The stereoselectivity of the interaction with muscarinic receptors of enantiomers of a series of chiral antagonists is receptor subtype dependent. There is no overall relationship between stereoselectivity and receptor affinity. Depending on the antagonist studied, receptor stereoselectivity may indeed reflect : (1) the weakening or loss of a single interaction involving one of the four groups bound to the asymmetric carbon; (2) steric hindrance preventing optimum interaction of the low affinity stereoisomer with the receptor; and/or (3) the inversion of the relative positions of two moieties of the ligand with similar structural and electronic properties i.e. comparable affinities for the two corresponding subsites in the receptor.

Although at least 5 different proteins (termed m1 to m5) have been identified as muscarinic receptor subtypes (Ref. 1 and references therein), only three of them can be discriminated to date in binding or pharmacological studies[2].

The following muscarinic receptor subtypes have been characterized :

1. Both human NB-OK1 neuroblastoma cell line receptors (in [3H]NMS binding studies[3]) and presynaptic rabbit vas deferens receptors (activated by 4-Cl-McNeil-A-343 in pharmacological studies[4]) show typical 'M$_1$' properties, comparable to the binding properties of the m1 receptor protein.

2. Rat heart (C) receptors (in [3H]NMS binding studies[5]) and guinea-pig atrial receptors[6] (in pharmacological studies) have typical 'M$_2$' selectivity, comparable to the binding properties of the m2 protein[1].

3. Binding sites with slow [3H]NMS dissociation rate in forebrain ('B' sites)[5], rat pancreas [3H]NMS-labelled binding sites[7], and guinea-pig ileum smooth muscle receptors[6] (functional studies) have very similar specificities. The binding properties of forebrain 'B' sites and pancreas sites are not identical (see below). It is quite possible that in the near future they will be subdivided into 'M$_3$' and 'M$_4$' subtypes. Since their binding properties are very similar to the binding properties of both m3 and m4 proteins, we shall hereafter call them 'M$_3$' receptors.

The M$_1$/M$_2$/M$_3$ nomenclature we adopted in this paper is that adopted for this symposium (see p. VII).

Why should we study receptor stereoselectivity?

The antagonist (and agonist) binding sites of muscarinic receptors are asymmetrical[8], and hence generally capable of discriminating optical isomers of chiral

drugs[8-16]. Thus analyses of binding and/or pharmacological properties of stereoisomers provide information about binding site geometry.

For the sake of simplicity, we shall hereafter call 'eutomer' the isomer with greater affinity for receptors and 'distomer' the lower affinity isomer[17]. This nomenclature, proposed by Lehmann[17] has the advantage of classifying drugs on the basis of their pharmacological and binding properties rather than on (somewhat arbitrary) chemical priority rules. The ability of receptors to discriminate between eutomers and distomers is manifest in their affinity ratios, also called 'eudismic ratio'[17]. The logarithm of this ratio is the 'eudismic
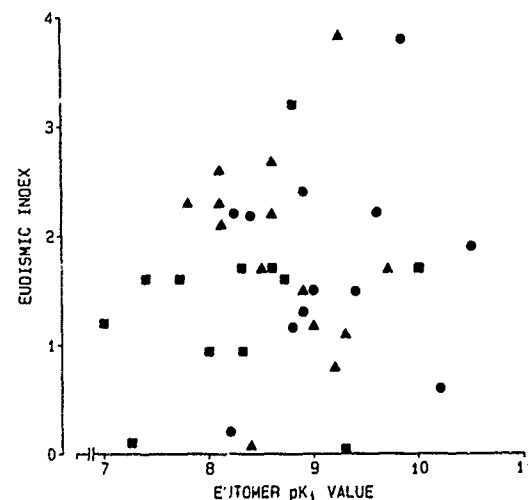

Fig. 1 The eutomer-pK$_i$ values of telenzepine[12], trihexyphenidyl, procyclidine, and benzetimide (dexetimide)[13], trihexyphenidyl methiodide, hexbutinol, p-fluoro-hexbutinol, hexbutinol methiodide, hexahydro-difenidol, tricyclamol, oxyphencyclimine, QNB and its methiodide (unpublished results) plotted as a function of their eudismic index for M$_1$ (●), M$_2$ (■) and M$_3$ (▲) receptors.
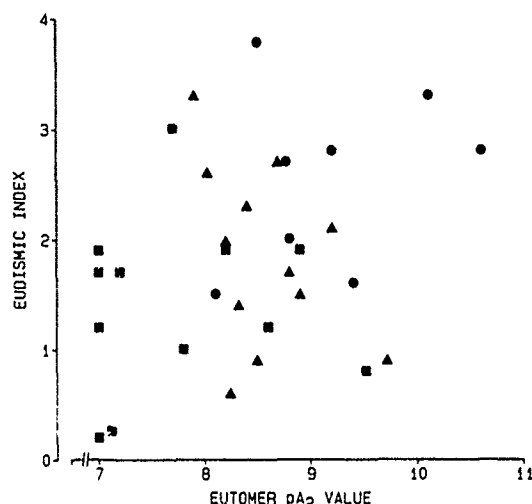
Fig 2 The eutomer pA_2 values of trihexyphenidyl and its methiodide[10], biperiden[11], phenglutarimide[15], hexbutinol, p-fluoro-hexbutinol, hexbutinol methiodide and hexahydro-difenidol (R. Feifel et al., unpublished results), procyclidine[9,21], tricyclamol[21], and QNB methiodide[18,19] plotted as a function of their eudismic index for M_1 (●), M_2 (■) and M_3 (▲) receptors

index'[17] (measured as the difference between eutomer and distomer pK_i or pA_2 values). It is directly proportional to the difference in the binding free energy of the two stereoisomers.

Several studies support the hypothesis that the eudismic indices at M_1, M_2 and M_3 receptors of antagonists such as procyclidine and sila-procyclidine[9], trihexyphenidyl and its methiodide[10], biperiden[11], telenzepine[12], hexahydro-difenidol (Feifel et al., unpublished data), benzetimide[13] or phenglutarimide[14] vary. The eudismic indices of a series of other antagonists were not significantly dissimilar at different muscarinic receptor subtypes (see for example Refs 18, 19).

What does the 'eudismic index' mean?

It has been suggested that the eudismic index correlates with the eutomer potency either when considering the interaction of a single drug with several receptor subtypes or that of various drugs with a single receptor[17].

We did not observe any correlation between eudismic index and affinity of the eutomer whether estimated in binding (Fig. 1) or functional (Fig. 2) studies.

A first and easy interpretation of this obvious lack of correlation is that the separation of the enantiomers of most antagonists used was incomplete. This hypothesis does not account for the lack of correlation shown in Figs 1 and 2. Indeed, in the case of samples prepared in our laboratories, the enantiomer purity was checked by analytical methods and found to be higher than 99%. Furthermore, if we compare, for example, the binding properties of benzetimide and QNB methiodide (Fig. 3), it is obvious that : (1) both eutomers had comparable affinities at three muscarinic receptor

subtypes; (2) the QNB methiodide eudismic index was much lower than that of benzetimide; and (3) these binding properties of (R)- and (s)-QNB methiodide were however sufficiently different to refute the hypothesis that its low eudismic index was due to incomplete eutomer and distomer separation. (R)-QNB methiodide did not discriminate between M_2 and M_3 receptors whereas (s)-QNB methiodide preferred M_2 over M_3 muscarinic receptor subtypes. The high optical purity of QNB and QNB methiodide enantiomers (over 99%) was corroborated by the finding that the affinity of (R)-QNB to M_2 receptors in rat heart was higher than that of (s)-QNB by about 100-fold (see below, Fig. 5).

These results suggest that the binding properties of two different molecules (i.e. the pure (R)- and pure (s)-enantiomers) had indeed been compared, and that muscarinic receptors, in several cases, discriminate very poorly the two enantiomers despite their high affinity for the eutomer (see for example Fig 3). This indicates that factors other than affinity are involved in the capacity of muscarinic receptors to discriminate between eutomers and distomers[9].

Three models are proposed to explain the stereoselectivity of muscarinic receptors for some antagonists and their implication for our understanding of drug–receptor interaction are discussed.

Stereoselectivity due to the loss of a single interaction

In the structure of benzetimide the four groups bound to the chiral centre have very different electronic
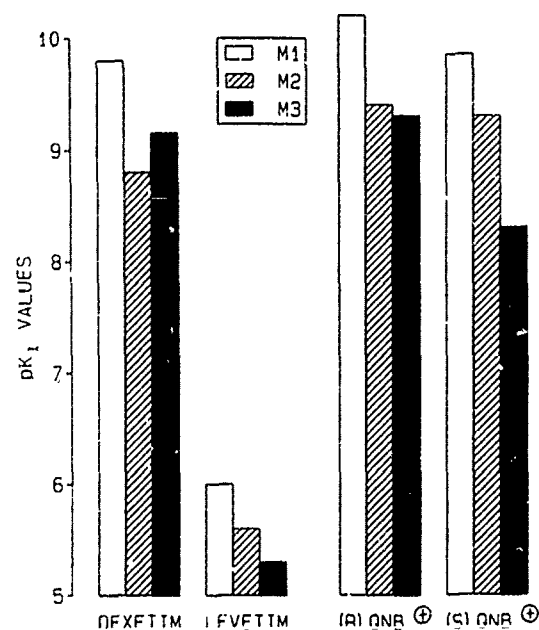

Fig 3. Comparison of the apparent pK_i values of dexetimide ((s)-benzetimide) and levetimide ((R)-benzetimide) and of (R)- and (s)-QNB methiodide (QNB ⊕) observed after 2 hours of incubation, using NB OK1, heart or pancreas homogenates (M_1, M_2 or M_3 sites, respectively)

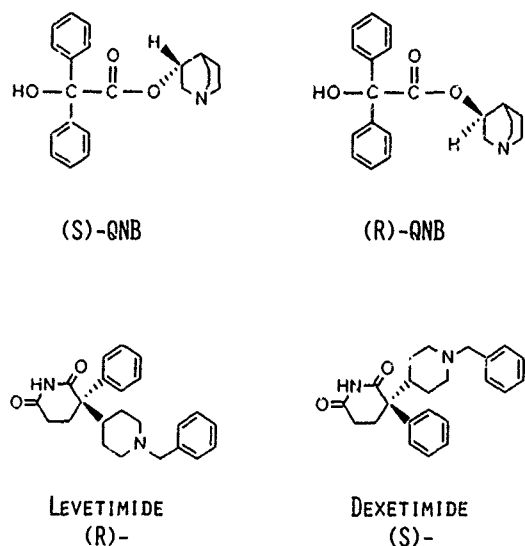(S)-QNB   (R)-QNB

LEVETIMIDE   DEXETIMIDE
(R)-   (S)-

Fig. 4 Chemical structures of dexetimide (or (s)-benzetimide), levetimide (or (R)-benzetimide), (R)-QNB and (s)-QNB

and structural properties (Fig. 4). If, when dexetimide (the eutomer) recognizes the receptor, the four groups specifically interact with receptor subsites, it is reasonable to expect that one of these groups will be incapable of recognizing the receptor in the distomer configuration. The very large eudismic indexes obtained in binding or pharmacological studies between dexetimide and levetimide confirm that the four moieties at the centre of chirality may interact strongly with the receptor. the difference between the eutomer and distomer binding free energies is at least 4.2 kcal/mol.

Stereoselectivity due to steric hindrance impeding distomer binding

When comparing (R)- and (s)-QNB binding properties we observed relatively low eudismic indexes

Fig 5 Comparison of apparent pK_i values of (R)- and (s)-QNB and of (R) and (s)-QNB methiodide (QNB⊕), obtained after 4 hours incubation with [3H]NMS at 25°C, in NB-OK1 (M_1 sites), heart (M_2 sites), striatum "B" (M_3 sites) or pancreas (M_3 sites) homogenates.

(considering the very high affinity of (R)-QNB) (Figs 4 and 5). N-Methylation of QNB decreased the affinity of the (R)-enantiomer but increased the affinity of the (s)-enantiomer; QNB methiodide bound with an even lower eudismic index than QNB at all receptor subtypes studied. The cardiac M_2 and neuroblastoma M_1 receptors were in fact virtually unable to discriminate between the (R)- and (s)-QNB methiodide enantiomers.

We would like to suggest nat, when the (R)-enantiomer of QNB recognizes ...uscarinic receptors, its protonated amino group is very close to the charge-neutralizing (carboxyl) group of the receptor. The resulting formation of a very strong ionic bond, would produce a high energy barrier for breaking this bond so that the corresponding dissociation rate of (R)-QNB is low. When (s)-QNB recognizes muscarinic receptors, its protonated amino group might be unable to get as close to the neutralizing group due to steric hindrance by the (s)-configurated quinuclidinyl group. Thus, the affinity of (s)-QNB is lower but the distance between the amino group of QNB and the neutralizing receptor group becomes large enough to accommodate a methyl group. (s)-QNB methiodide has, therefore, a greater affinity than (s)-QNB for most receptor subtypes.

TABLE I Kinetic constants of the interaction of QNB and QNB methiodide enantiomers with M_2 receptors

Antagonist	k_{on} ($10^8 M^{-1}$ min^{-1})	k_{off} (min^{-1})	$t_{\frac{1}{2}}$ (min)	pK_i
(R)-QNB	1 02	0.0092	75	10 0
(s)-QNB	1.11	0 60	1 2	8.3
(R)-QNB⊙	2 56	0 11	6 3	9 4
(s)-QNB⊕	5 81	0.25	2 3	9.4

The association (k_{on}) and dissociation (k_{off}) rate constants, half life of the complex and pK_i values of (R)- and (s)-QNB and of (R)- and (s)-QNB methiodide (QNB⊕) in rat heart (M_2) muscarinic receptors were calculated as described in Ref 20, using the equations described by Motulsky and Mahan to interpret the time dependence of competition curves
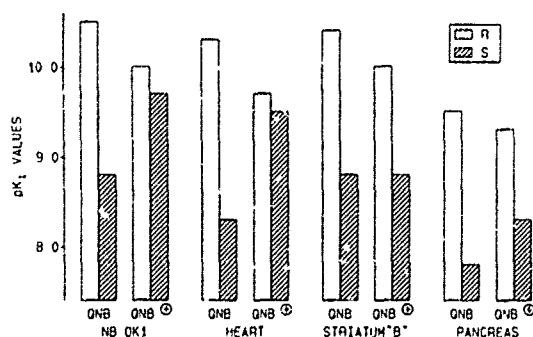
This hypothesis is supported by the analysis of QNB and QNB methiodide binding kinetics to cardiac M_2 receptors, as outlined in Table I. (s)-QNB had a markedly higher dissociation rate than (R)-QNB, a result compatible with weaker short-range interactions for the (s)-enantiomer. (R)- and (s)-QNB methiodides showed very similar, intermediate dissociation rate constants. The energy barrier for dissociating the (s)-QNB–receptor complex was increased by the presence of the N-methyl group.

It is very interesting in this respect that (s)-QNB methiodide displayed no higher affinity than (s)-QNB for the M_3 receptor subtype in striatum: the binding subsites on the muscarinic receptors for the quinuclidinyl group probably have a subtype-dependent shape or size. The QNB derivatives studied here appeared to recognize preferentially the striatum 'B' (M_3) binding sites, as opposed to pancreas (M_3) binding sites (Fig. 5)

TABLE II. Potency of procyclidine enantiomers, pyrrinol and hexahydroprocyclidine at M_2 receptors

Antagonist	pK_i values for rat striatal 'B' M_3 sites	pA$_2$ values for guinea-pig ileal M_3 sites
(R)-Procyclidine	8 1	8.04
Pyrrinol	7 2 (0.9)	6.91 (1.13)
Hexahydroprocyclidine	7.0 (1.1)	6 37 (1.67)
(S)-Procyclidine	6.0	5.46
Eudismic index	2.1	2.58
Expected eudismic index[a]	(2.0)	(2.80)

Measured at striatal M_3 receptors by binding studies (as described in Ref. 5), and at ileal M_3 receptors by functional studies (Ref 21 and unpublished) Values in parentheses are the differences between the drug pK_i or pA$_2$ values and the (R)-procyclidine pK_i or pA$_2$ values.
[a] Corresponds to the sum of the values in parentheses (see text for explanation)

suggesting that these binding sites belong to different subtypes. It is unfortunately not possible at present to correlate these binding properties with functional data as (R)-QNB behaves in pharmacological studies as a non-competitive antagonist[18,19]. We were also unable to assess the purity of the striatum 'B' subtype. Due to its very slow binding kinetics, (R)-QNB competition curves in pancreas were steeper than expected. The 'normal' competition curves obtained in striatum are compatible either with the existence of a single receptor subtype, or with the superimposition of two 'steep' competition curves at different receptor subtypes.

Stereoselectivity due to exchange of position of two groups in antagonist binding site

In contrast to benzetimide (Fig. 4), two substituents bound to the center of chirality of procyclidine (Fig. 6) possess comparable physicochemical properties. The cyclohexyl and phenyl moieties are both hydrophobic and incapable of promoting hydrogen or ionic bonds. To understand the forces responsible for the discrimination of procyclidine enantiomers by muscarinic receptors, we concentrated on the binding properties of procyclidine enantiomers and of two related achiral compounds: hexahydroprocyclidine and the diphenyl
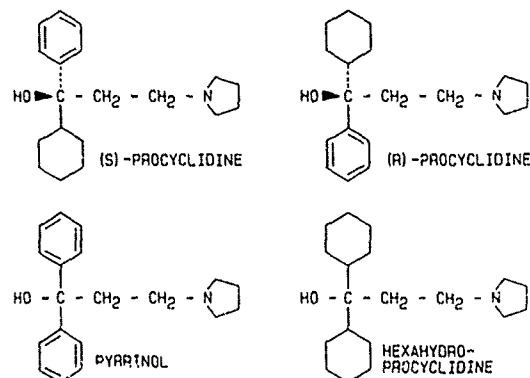
Fig. 6. Chemical structures of (R)- and (s)-procyclidine, pyrrinol, and hexahydroprocyclidine.

derivative, pyrrinol (Fig. 6). Their affinities for striatum 'B' sites are compared in Table I with pharmacological data obtained in guinea-pig ileum[21] and unpublished results (two systems with 'M_3' pharmacological properties). In binding and functional studies, (R)-procyclidine was more potent than the (s)-isomer. Replacing the cyclohexyl group by a phenyl group, or vice versa (from (R)-procyclidine to pyrrinol or to hexahydroprocyclidine), decreased the affinity by about one log unit. The distomer (s)-procyclidine had a lower affinity than the two achiral compounds, and the eudismic index of procyclidine binding to M_3 receptors was 2.1. As shown in Table II, affinity losses due to the replacement of the phenyl by a cyclohexyl and of the cyclohexyl by a phenyl group, measured on a log scale, were additive, the determined eudismic indexes being very similar to the calculated ones. This suggests that when (s)-procyclidine interacts with forebrain or ileum M_3 receptors, higher affinity is obtained by allowing the cyclohexyl moiety to interact with a 'phenyl-preferring' subsite, and the phenyl group with a 'cyclohexyl-preferring' subsite, rather than by allowing three of the four groups bound to the center of chirality to form optimal interactions with their respective subsites, the fourth group pointing away from the receptor.

The affinity (pK, values) and eudismic index correlated reasonably well in our four binding assays when comparing hexahydrodifenidol (HHD), trihexyphenidyl and procyclidine (Table III), three drugs with closely related structures. This suggests that the as-

TABLE III. Affinities and eudismic indexes of procyclidine, trihexyphenidyl and HHD

Antagonist	Eutomer pK_i and eudismic index values			
	NB-OK1 M$_1$	heart M$_2$	striatum B M$_3$	pancreas M$_3$
Procyclidine	8.4 (2 2)	7.4 (1 6)	8 1 (2 1)	7 8 (2 3)
Trihexyphenidyl	9 0 (2.5)	7 7 (1 6)	8.4 (2 6)	8 1 (2.6)
Hexahydrodifenidol	8.3 (2.2)	7 0 (1.2)	8.1 (2.1)	8 1 (2.3)

Affinities and eudismic index values (in parentheses) measured as described in Refs 3, 5 and 7 The correlation coefficient r between eutomer pK_i values and eudismic index values is 0 83

sociated eudismic indexes probably reflect an almost bio-isosteric exchange of the position of two moieties with similar (although not identical) structural and electronic properties. Since (s)-HHD (in contrast to (ʀ)-HHD) is nonselective, this suggests that the M_1, M_3 > M_2 selectivity of HHD[22] reflects poor binding of the phenyl/cyclohexyl moieties to cardiac M_2 receptor. On the other hand, trihexyphenidyl is M_1-preferring as compared to pancreas M_3 and M_2 receptors. The low eudismic index at M_2 receptors suggests that phenyl/cyclohexyl groups interact weakly with this receptor. Its large eudismic index at pancreas M_3 receptors, in contrast, implies that the M_1 > M_3 selectivity of trihexyphenidyl reflects a weaker binding of the basic center rather than the hydrophobic rings, to pancreas M_3 receptors.

Acknowledgment

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The design of full agonists for the cortical muscarinic receptor

John Saunders and Stephen B. Freedman

In the present study we describe a novel series of oxadiazole based tertiary amines which include the most efficacious and potent muscarinic ligands known. These compounds possess physicochemical characteristics which enable rapid equilibration into the CNS and are able to fully activate cortical muscarinic receptors. Data obtained from this series have allowed us to propose a pharmacophoric model which distinguishes high and low affinity state binding. This in turn has led us to suggest that agonists and antagonists may bind at two independent sites on the receptor protein and to speculate on the steps putatively involved in agonist-induced receptor activation.

For the past four years we have been involved in the design of cholinomimetic agents which would be capable of penetrating into the CNS and displaying high efficacy at cortical muscarinic receptors. In the cerebral cortex, those muscarinic receptors which are coupled to the turnover of phosphatidylinositol[1] (PI) appear to have only limited receptor reserve. It is now known that there are at least three subtypes, m1, m3 and m5, which may be capable of positively stimulating PI hydrolysis[2].

Agonists such as pilocarpine and RS-86 are unable to produce a response of greater than 15% of that seen with the low affinity but high efficacy agonist, carbachol. Prior to the discovery of the novel compounds described in this report, it was believed that only quaternary ammonium based agonists such as carbachol, muscarine and oxotremorine-M, possessed sufficient intrinsic efficacy to maximally stimulate cortical PI hydrolysis. Such agents would be expected to have only very limited capacity to penetrate into the CNS.

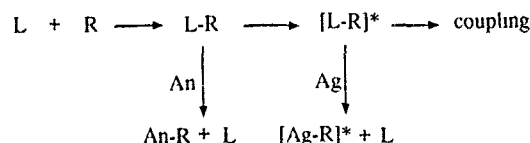### Efficacy at the cortical muscarinic receptor

We have previously described[3-5] a two step binding assay to measure affinity and also predict cortical efficacy from the antagonist–agonist binding ratio in rat cortical membranes (Fig. 1); that is the NMS/OXO-M ratio. The log of this ratio has been shown to correlate with the ability of the ligand to stimulate the hydrolysis of cortical PI. Four broad categories of muscarinic ligands can be defined according to their efficacy as estimated from this ratio: antagonists show equal affinity in both binding assays and thus have ratios close to unity, weak partial agonists have low ratios between 10–200, partial agonists display intermediate ratios of 200–800 whereas, at the other end of this continuum, full agonists display a ratio in excess of 800.

Discovery of oxadiazole based muscarinic ligands

Starting from arecoline[6], our objectives were three-fold: (1) to design an agonist with at least a ten-fold and preferably up to 100-fold increase in receptor affinity relative to arecoline (see Table I); (2) to improve on the

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physicochemical properties of that compound (i.e. remove the hydrolytically susceptible ester bond) and (3) to increase efficacy to that of a full agonist while maintaining the ability to rapidly equilibrate into the CNS. This latter point was thought to be the most problematic since established dogma was that the quaternary ammonium entity was an obligatory feature for all full agonists at the cortical site.

It had been observed that replacement of the ester functionality in benzodiazepine esters by oxadiazole invariably produced an increase in efficacy as witnessed by an increase in the GABA shift[7]. In fact the GABA shift may be compared to the NMS/OXO-M ratio in predictive value for efficacy. The first compound made involved this direct replacement into arecoline and afforded compound 1 (Table I) which showed a modest improvement in affinity but without the anticipated increase in efficacy. However, the compound does represent a hydrolytically stable arecoline-like agonist. Almost simultaneously, a series of quantum mechanical calculations were undertaken aimed at trying to understand more precisely the distribution of electrostatic charge on a quaternary methylammonium group. It was concluded that the spherically symmetrical distribution of charge should be best mimicked by a protonated 1-azabicyclic system such as is found in the quinuclidine framework. Thus the second new compound, 2, provided the jump in affinity desired but

$$L + R \longrightarrow L\text{-}R \longrightarrow [L\text{-}R]^* \longrightarrow \text{coupling}$$

$$\text{An} \downarrow \qquad\qquad \text{Ag} \downarrow$$

$$\text{An-R} + L \qquad [\text{Ag-R}]^* + L$$

$$\text{PREDICTED EFFICACY} \; \alpha \; \frac{K_i(\text{NMS})}{K_i(\text{OXO-M})}$$

Fig. 1 Antagonist–agonist binding ratio in cerebral cortex as a measure of cortical efficacy L = radioligand (either [³H]-NMS or [³H]-OXO-M), R = receptor, An = antagonist, Ag = agonist, * = activated state of receptor

TABLE I. *In-vitro* binding data for arecoline and novel oxadiazole-based ligands

Compound	Binding data [K_a(app) (μM)]		
	[³H]NMS[a]	[³H]OXO-M[b]	ratio[c]
Arecoline	6 2 (5 1–9 0)	0 011 (0 0098–0 012)	560
1	1 8 (1 0–2 3)	0 0046 (0 0035–0 0063)	390
2	0 44 (0 39–0.48)	0 00090 (0.00052–0 0013)	490

Values determined by displacement of tritiated ligand from rat cortical homogenates, expressed as affinity constant [K_a(app)] corrected for ligand occupancy using the Cheng and Prusoff equation. Each value is the geometric mean of at least three determinations performed on separate occasions, for each determination assays were performed in triplicate using, typically, 6–10 concentrations Ranges of values given in parentheses
[a]Displacement of [³H]N-methylscopolamine, [b]displacement of [³H]oxotremorine-M; [c]ratio of K_a(app) for NMS to K_a(app) for OXO-M.

again with little change in efficacy. Nevertheless, this result prompted further development of the quinuclidine series.

Progressive removal of the heteroatoms by making oxazoles and furans (Table II) caused a drastic reduction in efficacy, primarily by loss in binding to the high affinity state of the receptor, while leaving low affinity state binding relatively unchanged. The exact position of the heteroatoms was also important since isomeric oxadiazoles (e.g. 6) showed both lower affinity and efficacy. Noting that the arrangement of heteroatoms in 2 was optimal both for affinity and efficacy, changes to the oxadiazole substituent were next explored (Table III). Compounds 2 and 7–9 indicated that efficacy fell off very rapidly with increasing bulk lipophilicity roughly in the vicinity of where the methyl group of acetylcholine would be found. Ethyl for example had reduced efficacy similar to that of a weak partial agonist. The substitution of the diphenylhydroxy group ('borrowed' from QNB) produced an antagonist with affinity equal to most classical antagonists. The data also gave the first clues as to how efficacy may be improved since it suggested that even the methyl group in this series may be too lipophilic.

Recalling that an amino group in carbachol replaces the methyl group of acetylcholine, this substitution was studied in the oxadiazole series. The resulting compound, 10, showed an increase in efficacy through improved binding to the high affinity state of the receptor. Several pairs of methyl and amino compounds revealed that this improvement in efficacy with

amino was quite general. Increasing lipophilicity by N-methylation to give the monomethylamino-, 11, and dimethylamino-oxadiazole, 12, again resulted in loss of efficacy. Finally, the chloro substituent, both isosteric and isolipophilic to methyl not surprisingly yielded a compound, 13, with a profile closely similar to 2.

At this stage, reduction in the bulk lipophilicity of the quinuclidine ring was studied. The simplest conceptual change was to remove a carbon atom from one of the bridges in quinuclidine since this would allow lipophilicity to be lowered while maintaining a similar geometry of the pharmacophore. This proved to be the definitive change in improving both affinity and efficacy and has provided agonists (Table IV) with affinity and efficacy comparable to or greater than that of all classical quaternary ammonium based ligands. In contrast to quinuclidine, since the resulting azabicycle is no longer symmetrical, there are two diastereomeric forms of the molecule which we have labelled *exo* and *endo*. In all cases, it is the *exo* form, the form with the oxadiazole ring directed towards the one carbon bridge, which is the more active. The figure of 0.040 nM for 16 is an underestimate of the compound's true affinity for the OXO-M labelled state because of ligand depletion in the binding assay. When the determination was repeated at lower membrane dilutions, the affinity was increased to give a ratio in excess of 1000.

In-vitro studies

Four of the compounds discussed above were the subject of a more detailed biological characterization. The methyl oxadiazole, 2, showed a 10-fold increase in affinity compared with arecoline but had only a limited ability to stimulate PI turnover with a maximum response of 18% of that seen with carbachol (Table V). This response was similar to that seen with arecoline though it possessed some 3-fold higher potency. The amino oxadiazole, 10, with a significantly higher NMS/OXO-M ratio, produced a dose dependent stimulation of cortical PI turnover with a maximal response at 1 mM of about 70% of that seen with carbachol.

TABLE II *In-vitro* binding data for quinuclidine-based ligands

Compound	HET	Binding data [K_a(app) (μM)]		
		[³H]NMS[a]	[³H]OXO-M[b]	ratio[c]
2		0.44 (0.39–0 48)	0.00090 (0 00052–0.0013)	490
3		1 4 (1 2–1 7)	0.027 (0 022–0.033)	52
4		1 4 (1 1–1 8)	0 036 (0 030–0 045)	39
5		1.4 (0 95–1 9)	0 057 (0 041–0 067)	15
6		7 5 (5 4–12)	0 060 (0.054–0 071)	130

Footnotes as defined in Table I.

TABLE III. *In-vitro* binding data for substituted oxadiazoles

Compound	R	[³H]NMS[a]	[³H]OXO-M[b]	ratio[c]
			Binding data [K_a(app) (μM)]	
2	CH₃	0 44 (0 39–0.48)	0.00090 (0 00052–0 0013)	490
7	CH₂CH₃	0.096 (0.08–0.11)	0 0056 (0 0047–0.0073)	17
8	CH₂Ph	0.070 (0.049–0 10)	0 021 (0.014–0 031)	3 3
9	C(OH)Ph₂	0 00012 (0 0001–0.00012)	0 000067 (0.00015–0 000037)	1 8
10	NH₂	0 60 (0 44–0.70)	0.00050 (0.00035–0 00067)	1200
11	NHCH₃	2.0 (1.2–3 5)	0 053 (0 021–0 17)	38
12	N(CH₃)₂	0 20 (0.13–0 37)	0.021 (0 014–0.025)	9.5
13	Cl	0.19 (0 18–0 21)	0 00048 (0.00041–0 00054)	400

Footnotes as defined in Table I.

When the quinuclidine base was replaced with a 1-azanorbornane system the resulting exo methyl and amino oxadiazoles (*14* and *16* respectively) displayed a 5–10-fold increase in affinity compared with the quinuclidine and also an increase in the predicted efficacy in both cases. Compound *14* stimulated cortical PI turnover with a maximum response of 90% of that seen with carbachol whereas the amino analogue *16* was amongst the most efficacious muscarinic agonists known. The magnitude of the PI response at about 150–170% was equivalent to that seen with acetylcholine (EC50=30 μM), though this compound was 100-fold more potent. The responses of these compounds were inhibited by the muscarinic antagonist atropine (1 μM).

In-vivo characterization

Muscarinic agonists produce a wide variety of physiological effects when administered to rodents, including central effects such as hypothermia and peripheral effects such as salivation. The potency of arecoline in these models was very weak (Table VI) with doses in excess of 10 mg/kg being used due to the labile nature of the ester linkage present in arecoline. In contrast, the oxadiazole derivatives were considerably more active *in vivo*, with the two quinuclidine derivatives producing cholinomimetic effects at doses up to 1000-fold less than arecoline (Table VI). The two azanorbornane derivatives, *14* and *16*, showed even higher potency with *14* producing central cholinomimetic effects at doses of less than 1 μg/kg. These compounds are some of the most potent centrally active cholinomimetics known.

The two methyl oxadiazoles *2* and *14* were interesting in that they demonstrated peripheral and central effects at similar doses, implying that both compounds

possessed good penetration into the CNS. This was examined in more detail in an *ex-vivo* binding assay[8]. In this assay compounds were tested for their ability to displace the *ex-vivo* binding of the potent muscarinic agonist [³H]OXO-M in whole mouse brain homogenates. The binding observed in saline treated animals was compared with that from mice previously treated with novel cholinomimetics and the inhibition of specific binding by the latter obtained by comparison. An estimation of the penetration of each compound

TABLE IV Binding data for azanorbornane-based oxadiazoles

Compound	[³H]NMS[a]	[³H]OXO-M[b]	ratio[c]
	Binding data [K_a(app) (μM)]		
2	0 44 (0 39–0 49)	0 00090 (0 00052–0 0013)	490
14	0.10 (0 076–0 21)	0 000i0 (0 000055–0.00015)	1000
15	3.6 (1.9–5.9)	0.0021 (0.0015–0 0027)	1700
16	0 032 (0 023–0 040)	<0 000040 (0 000034–0 000045)	>1000
17	1.8 (1 4 –2.1)	0 0014 (0 0011–0.0015)	1300

Footnotes as defined in Table I

TABLE V. *In-vitro* characterization of oxadiazole muscarinic receptor agonists in rat cerebral cortex

Compound	NMS:OXO-M ratio[a]	Phosphatidylinositol turnover EC50 (µM)[b]	% max response[c]
Arecoline	560	16	18
2(L-658,903)	490	5	16
10(L-660,863)	1200	3	68
14(L-670,548)	1000	0 25	90
16(L-670,207)	>1000	0 25	170

[a]Ratio of K_a(app) for NMS to K_a(app) for OXO-M [b]Potency of compound in eliciting breakdown of labelled inositol phospholipids. Each value calculated from 2–5 independent determinations [c]% Maximum response expressed as % response to 1 mM carbacol, included in all experiments.

into the CNS was also made by comparison with a calibration curve of compound added to homogenates from saline treated animals. In this assay arecoline was very weak with a potency of 220 mg/kg measured at 30 min following administration (Table VI). This low potency and the poor penetration obtained for arecoline is a reflection of its poor biological availability and short duration of action. In contrast, the four oxadiazoles were up to 30000-fold more active than arecoline with the methyl oxadiazole 14 being the most potent (ED50 0.0069 mg/kg). Because of the dilution of tissue in the binding assay the *in-vivo* dose required to yield 50% occupancy of brain muscarinic receptors is considerably lower than this figure. Both of the methyl oxadiazoles showed good CNS penetration in the *ex-vivo* assay with amounts in the CNS calculated to be 1.9 and 3.8% of the administered dose. These values

are some 10-fold higher than those observed with the amino oxadiazoles and represent very high penetration, since the weight of the mouse brain as a proportion of the total available body weight is in the order of 2–5%.

A pharmacophoric model to distinguish high and low affinity state binding

Taken together this information has allowed the development of a simple binding model (Fig. 2). As has been obvious for some time, all muscarinic ligands require the presence of a cationic head group which, by analogy with the β-receptor[9], is involved in an electrostatic interaction with an Asp residue on the receptor protein and this is seen as the primary interaction. Sites for secondary interactions, and those which allow us to differentiate between agonists and antagonists, are provided for by the oxadiazole moiety. From the structure–activity discussion presented above, it was concluded that two H-bond acceptor sites on the ligand are necessary for full agonist behaviour and furthermore, these sites are localized in the vicinity of the two ring nitrogens[10]. The role of the electron-donating amino group is simply to potentiate the H-bonding capability of the ring nitrogens. More precisely, efficacy is correlated directly with the negative electrostatic potential adjacent to the ring nitrogens.

Thus two H-bond acceptors are necessary for binding with high affinity to the agonist binding site with little or no dependency on lipophilic binding. In contrast, either or both of these interactions are relatively unimportant for binding to the low affinity state. Instead, increased reliance is placed upon lipophilic binding in the vicinity of the acetylmethyl group of the endogenous ligand, acetylcholine, and this characterizes antagonist behaviour.

These differences may be summarized as follows: antagonists and agonists distinguish different binding loci on the receptor although the cationic head group is

TABLE VI. *In-vivo* characterization of novel oxadiazole muscarinic receptor

Compound	Salivation FS100[a]	Hypothermia ED50[b]	Ex-vivo binding	
			ED50[c]	% penetration[d]
Arecoline	66	13	220	0 0035
2(L-658,903)	0.015	0 015	0.21	1 9
10(L-660,863)	0.007	0 09	0.65	0 13
14(L-670,548)	0.0007	0 0006	0.0069	3 8
16(L-670,207)	0.0015	0.0030	0 064	0 24

[a]Dose of agonist (mg/kg) required to produce 100 mg of saliva during the experimental 10 min period in 25 g BKTO male mice [b]Dose of agonist required to produce 50% of the maximum fall in core body temperature. [c]Dose of compound required to inhibit the specific binding of [3H]OXO-M (0.6 nM) by 50%. [d]Penetration of each compound was estimated by comparing inhibition of binding with a calibration curve constructed by adding known amounts of test compound to the homogenates from saline-treated animals. Using this calibration curve, the amount of test compound in the original homogenate was estimated Penetration of each compound was calculated by comparing the amount of compound in the brain and dividing this by the total amount administered and multiplying by 100 Data are the mean of 3–5 independent determinations.
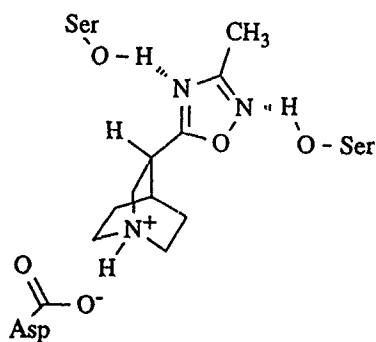
Fig. 2. Hypothetical binding site for muscarinic ligands showing the presence of the primary Coulombic interaction between the protonated amine (or quaternary ammonium group) and an Asp residue from the receptor In addition, agonists utilize two H-bond interactions, possibly provided by Ser residues, whereas antagonists require lipophilic binding in the region of the oxadiazole methyl group.

an essential pharmacophoric element for all ligands. Full agonists utilize two H-bonding interactions (acceptors) to bind to the receptor and, in general, are small, hydrophilic species. They also require some degree of conformational flexibility to participate in the mutual conformational changes that accompany receptor activation. Antagonists require maximally one, and possibly no, H-bonding sites and instead utilize hydrophobic binding energy to stabilize binding to the receptor. Accordingly, they are large molecules with some region of bulk lipophilicity. Although it is proposed that there are two distinct binding loci on the receptor, only one of these sites is accessible to antagonists because of their chemical and physico-chemical properties.

Agonist-induced receptor activation

The ligand binding model depicted in Fig. 2 permits speculations on the sequence of events in agonist-induced receptor activation that is consistent with our ligand docking experiments to a 3-dimensional model of the muscarinic receptor. This model is based on John Findlay's rhodopsin model[11] in which the most prominent feature is the seven transmembrane helical domains which encompass the ligand binding site. By analogy with the β-adrenergic system, it is to be expected that two functional aspartic acid (Asp) residues play a role in ligand binding. For the β-receptor, Asp-113 has been shown to be the primary recognition site for all ligands and, more interestingly, a second Asp, Asp-79, seems to be involved in agonist but not antagonist binding. These residues correspond to Asp-105 and Asp-71 respectively on the ml receptor protein.

As shown in Fig. 3, the ligand, irrespective of its efficacy, binds initially to the low affinity state of the receptor, that is with Asp-105 found towards the extracellular end of helix 3, in the first equilibrium process. The ligand has been purposely located above the Asp to indicate that, although the protonated amine function is electrostatically bound to this Asp, the other

end of the molecule is directed towards the surface of the receptor. This would imply that, for antagonists, the large lipophile is bound in lipophilic pockets on the receptor close to its surface. Only if the ligand is an agonist is the second equilibrium process achieved with the ligand moving down into the binding channel to take up a new position with the oxadiazole now directed down into the protein but with the cationic head group still bound to Asp-105. Stabilization of binding at this stage can no longer rely on lipophilic binding since the oxadiazole ring and its substituent find themselves in a highly hydrophilic climate. H-bonding interactions are used at this stage and also later to assist in the shuttling process by which the agonist becomes attached to a lower Asp residue, Asp-71, buried midway down helix 2. It is this sequence of events that distinguishes agonists from antagonists and results in mutual changes in conformation in both the agonist and receptor which are associated with receptor activation. This has two consequences. First, we can reinterpret the NMS/OXO-M assay and state that antagonists displace radio-labelled agonist binding only indirectly by continually disturbing the equilibria in favour of low affinity state binding. Second, it provides a qualitative description of partial agonists whose efficacy will depend on the exact
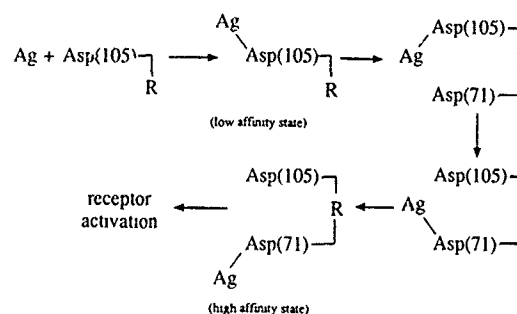
Fig. 3 Sequence of events putatively involved in receptor activation following ligand binding. Each step should be seen as an equilibrium process. Asp105 is located towards the extracellular surface on helix 3 and is the first recognition point for all muscarinic ligands Only small, hydrophilic molecules (Ag, agonists) have access to the Asp71 buried almost at the midpoint of helix 2.

position of these equilibria. For full agonists, this equilibrium will almost entirely favour high affinity state binding.

In conclusion, these studies have led to some of the most efficacious and potent agonists known which are capable of penetrating into the CNS. The structure–activity trends have improved our understanding of structural properties which influence efficacy and this in turn has led to a working hypothesis for the steps involved in receptor activation.

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Cholinergic mechanisms in pain and analgesia

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There is now substantial evidence that acetylcholinesterase inhibitors and muscarinic receptor agonists increase the pain threshold after both systemic and spinal administration. In rats, physostigmine gave a significant dose-dependent increase in latency times in the tail immersion test following intrathecal administration. The effect was antagonized with atropine. Neostigmine gave more prolonged latencies as did the muscarinic receptor agonist carbachol. Spinal cholinergic pathways for antinociception interacted with the spinal opioid and adrenergic nerve tracts. No cross-tolerance to the selective α_2-adrenoreceptor agonist guanfacine or to morphine was seen in rats tolerant of spinal carbachol antinociception. The mechanism of spinal cholinergic antinociception is not known but a muscarinic interneuron may explain the interactions with other neurotransmitters. Clinically, the centrally active cholinesterase inhibitor physostigmine has been shown to give postoperative pain relief although of short duration. Severe neurogenic pain has been successfully treated with physostigmine or distigmine.

More than '. '. have now elapsed since the first observation . · .e that the cholinesterase inhibitor physostigmine increased the pain threshold in humans[1]. Since then there have been many reports describing the antinociceptive action of both cholinesterase inhibitors and cholinergic agonists (for review see Refs 2 and 3). It has also been reported that physostigmine increased the threshold for pain elicited by radiant heat to the forehead of human subjects[4]. Experimental pain research on these drugs was actively pursued before the discovery of the endogenous opioid peptides but has since then become less active. However, in recent years research on cholinergic mechanisms for pain and analgesia has had a renaissance. For example, studies have revealed an antinociceptive effect of physostigmine and other cholinomimetics i.e. oxotremorine, in different animal species[5]. Centrally administered acetylcholine has also been found to cause antinociception, which was proposed to depend on the activation of descending pathways[6].

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The aim of this article is to review the present knowledge of cholinergic mechanisms for pain and analgesia. Experimental studies in animals using different pain models will be discussed as well as effects on antinociception after spinal application of cholinesterase inhibitors and cholinergic agonists. Interaction with spinal cholinergic antinociception using drugs active in other pain modulatory pathways will be described as well as autoradiography on the localization of cholinergic tracts in the spinal cord. Clinical studies using cholinomimetic drugs are at a very early stage, but interesting clinical results have been reported, particularly in patients with neurogenic pain states.

Animal studies
Antinociceptive studies in animals
The tests for antinociception in animal models usually involve the study of the latency time of a motor response to thermal, mechanical, electrical or chemical stimuli considered to cause pain[3]. A delay in a withdrawal response is not necessarily a consequence of analgesia, but may be caused by a motor effect or excessive sedation, making the animal unable to react. The effect on motor performance is particularly crucial in studies of spinal cholinomimetic drugs due to the

existence of cholinergic pathways in the α-motor-neuron area of the spinal cord. It is therefore hazardous to extrapolate conclusions from experiments on nociceptive thresholds in animals to the complex pain behaviour in humans. Nevertheless, studies in animals are useful for investigating compounds of potential interest as analgesic agents, and for determining their mechanisms of action. Several models for antinociceptive testing have been used throughout these studies. In the hot plate test, the response latency for a rat to lick its hindpaw after having been placed on a hot metal surface is recorded. This response is considered to require the involvement of both spinal and supraspinal structures. The tail flick test, i.e. the response latency of the animal to withdraw its tail from radiant heat produced by a beam of light focused on the tail, is suggested to reflect a segmental spinal reflex. Thirdly, the tail immersion test, where the tail of the rat is immersed in hot water, produces a lower intensity of stimulus which increases more slowly as compared to the tail flick test. This may represent a more physiological nociceptive stimulation allowing the experience of fast and slow pain, as well as endogenous modulation of the afferent stimuli.

Antinociception induced by spinal acetylcholine esterase inhibitors

Physostigmine. In studies carried out in our laboratory, intrathecal administration of physostigmine to rats produced a dose-related increase in response latency in the tail immersion test in the dose range 1–20 μg[7]. A statistically significant increase in latency time as compared to placebo was measured following 15 μg of

*Fig 1. Tail immersion latencies (ordinate s, mean ± s e) after intrathecal administration of physostigmine 15 μg (●) compared with clonidine 10 μg and physostigmine 15 μg (▲). n = 6 in both treatment groups. *p = 0.05 Taken from Ref. 8 with permission from Acta Anaesthesiol Scand.*

intrathecal physostigmine (Fig. 1). The effect of spinal physostigmine was of short duration and no antinociceptive effect was seen after 90 min. The antinociception was attenuated after intrathecal administration of the muscarinic receptor antagonist atropine. This indicates that spinal antinociception in the rat is mediated by muscarinic cholinergic receptors[7]. On the other hand, intrathecal physostigmine did not produce any change in the latency times in the hot plate test[7].

THA. The reversible choline esterase inhibitor 9-aminotetrahydroacridine (THA) has been used in clinical anesthesia as a ventilatory stimulant and for the prolongation of neuromuscular blockade of succinylcholine[8]. Studies have also shown THA to be effective as an adjunct to morphine in the treatment of intractable pain states[9]. It is a well-known cholinesterase inhibitor with central effects[10]. A degree of nicotine receptor agonist activity has also been suggested[11] and THA has recently been reported to antagonize the N-methyl-D-aspartate (NMDA) receptor complex[12]. The effects on these different receptors in the spinal cord is not known. In our studies in the rat THA did not produce any increase in latency times in the tail immersion test following spinal adminstration (Fig. 1) On the other hand, a significant decrease in total motor activity, rearing and locomotion was measured[13]. The motor effects were attenuated with atropine whereas the nicotine receptor antagonist mecamylamine had no effect on the decreased motor effects. This suggests that these effects were mediated primarily by muscarinic receptors.

Neostigmine. Neostigmine is a reversible cholinesterase inhibitor containing a quaternary ammonium functional group and is slowly eliminated from the intrathecal space probably due to its hydrophilic character. Consequently, intrathecal neostigmine in the rat gave rise to an increase in latency times that was of much longer duration than that for physostigmine. After three hours a profound effect could still be seen. The effects were promptly antagonized by atropine[7]. The long duration of effect following spinal administration was also evident using the hot plate and the tail flick tests (Post, C. et al., unpublished).

Antinociception by carbachol

Carbachol is a quaternary ammonium compound with agonistic effects on muscarinic receptor. Owing to the hydrophilic properties of carbachol it may a have a long duration of action after intrathecal administration. A dose-dependent increase of nociceptive reaction times was evident following spinal administration of carbachol in the dose range 2.5–15 μg (Ref. 14). At doses of 20 μg and above motor impairment was pronounced. The antinociceptive effect was antagonized with atropine, and with either pirenzepine or AF-DX 116 (Ref. 14), which are selective M_1 and M_2 muscarinic antagonists, respectively. Spinal analgesia by carbachol in the rat is at present thought to be mediated through both M_1 and M_2 muscarinic receptors. However, a selective M_1 receptor agonist might be advantageous since it would produce spinal analgesia without motor impairment[14].
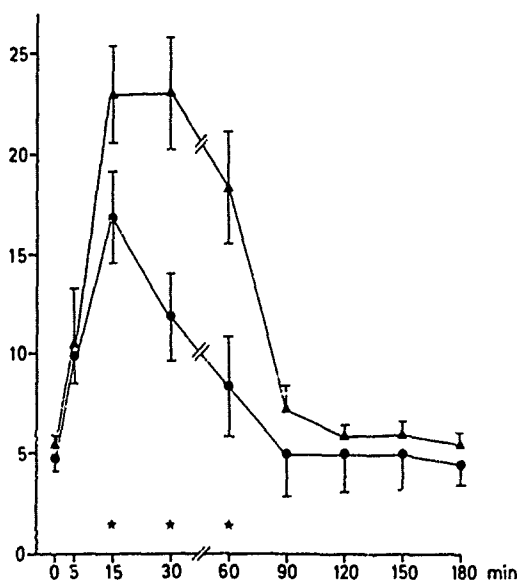
Other muscarinic receptor agonists have been tested for antinociceptive effects after spinal administration in the rat. High doses of the muscarinic receptor agonist McNeil-A-343 produced very prolonged latency times in the tail immersion test. Pilocarpine produced only a small increase in tail immersion latencies together with vigorous scratching in most animals. The scratching effect was attenuated with pirenzepine. (Gillberg, P.G. et al., unpublished). Others have shown antinociceptive response of other cholinomimetics e.g. oxotremorine after spinal doses to the rat[15].

Behavioral effects of nicotinic receptor agonists given spinally

The behavioral effects including antinociception, locomotion, rearing and total activity have been evaluated after intrathecal administration in the rat of nicotine and the nicotinic receptor agonist cytisine[13]. No significant increase in latency times in the tail immersion test was observed after nicotine whereas cytisine caused a small increase. Cytisine reduced total activity and locomotion shortly after the dose was given, whereas nicotine did not affect these parameters. The administration of nicotine and cytisine was also associated with aversive effects such as gnawing and vocalization. These effects were completely blocked by the nicotinic receptor antagonist, mecamylamine[13]. In summary, nicotinic cholinergic receptors do not seem to be involved in spinal antinociception. However, the blockade by mecamylamine of the aversive effects of nicotine and cytisine may indicate a role for nicotinic receptors in afferent sensory transmission[13].

The architecture of the spinal cord studied with autoradiography

Autoradiographic studies have demonstrated the existence of muscarinic receptors in substantia gelatinosa of the spinal cord[16-18] as well as a cholinergic interneuron located in the dorsal horn S19, a key locus for the transmission of pain in the spinal cord. In the dorsal horn, the muscarinic receptors are of M_1 and 'non-M_1' types in about equal numbers[16,17]. In the α-motor neuron area muscarinic receptors are represented as well and are predominantly 'non-M_1'[16,17]. Lesion studies indicate that muscarinic receptors in the spinal dorsal horn have a localization on the nerve terminals of the primary afferent, since lesioning of the primary afferent by dorsal rhizotomies leads to a rapid loss of muscarinic receptors in this area[18]. Further evidence for a presynaptic localization on the afferent neuron was that the number of cholinergic receptors in the spinal dorsal horn was unaffected after hemisections of the spinal cord[18]. It is thus not likely that the muscarinic receptors are located presynaptically on descending spinal neuronal terminals.

In addition to muscarinic receptors, enkephalinergic, noradrenergic[20] and serotoninergic[21] terminals have been shown to be present in the spinal dorsal horn Cholinergic receptor agonism may have a direct effect on spinal antinociception but seems also to interact with these other pathways in the spinal cord.

Nicotinic receptors have been localized using α-bungarotoxin to both the ventral and dorsal horns of the rat
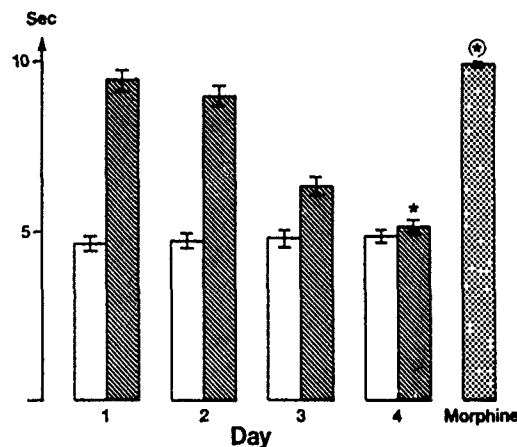
Fig 2 Antinociceptive effects (mean ± s.e., n = 6) measured as tail immersion latencies (ordinate) in the rat following twice daily intrathecal injection of carbachol 15 μg (solid bars) and saline (open bars) for four days. Thereafter the carbachol-tolerant rats were given an injection of morphine 10 μg and the tail immersion latency was measured.

spinal cord[18]. Acetylcholine binding to nicotinic receptors in man and cat on the other hand, has only been found in the dorsal horn and around the central canal, lamina X (Ref. 21). These regions transmit sensory information to the brain and receive terminals from the descending pain inhibitory tracts. As indicated, nicotinic receptor-mediated effects after intrathecal doses are probably not of significance for antinociception but might be of importance for other modalities of afferent sensory transmission.

Interaction with the spinal cholinergic pathways

Using the tail immersion test in the rat an additive antinociceptive effect was produced after intrathecal administration of physostigmine and the adrenoceptor agonist clonidine (Fig. 2)[7]. The antinociception induced by physostigmine was attenuated with the adrenoceptor blocker, phentolamine. Conversely, atropine attenuated the increased tail immersion latencies produced by clonidine[7]. Furthermore, in animals pretreated intrathecally with 6-hydroxydopamine leading to a selective lesioning of noradrenaline terminals, intrathecally injected physostigmine did not produce any nociceptive response[7].

Analgesia induced by intrathecally administered morphine is attenuated with atropine[22]. Thus, a complex system of interaction of spinal pathways in the processing of pain has to be considered. At present, the organization of these interactions is not known. One possible explanation for the interaction between spinal noradrenergic and cholinergic spinal tracts is the existence of a cholinergic interneuron, with possible relevance to antinociception, located in the dorsal horn[19]. This cholinergic interneuron may hypothetically receive excitatory input from descending noradrenergic pathways involved in the modulation of the transmission of nociceptive input. This interneuron
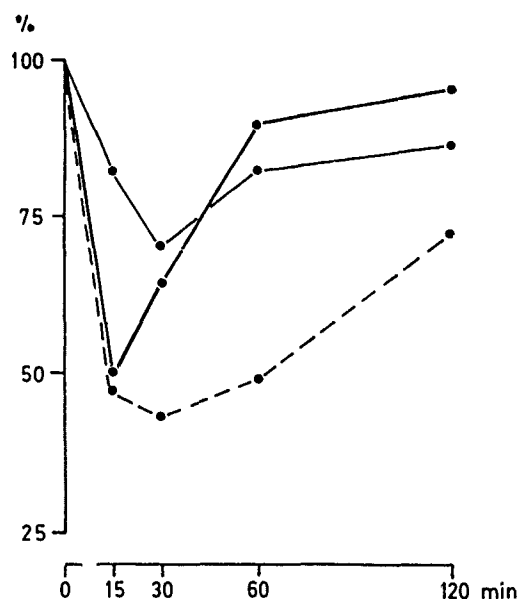
Fig 3 Analgesic effect in the early postoperative period expressed as the relative change of pain (ordinate) assessed by the patient in the early postoperative period following intravenous physostigmine salicylate 2 mg (——), pethidine chloride 50 mg (– – –) and placebo (——) n = 20 in each treatment group. Reproduced from Ref. 27 with permission from Acta Anaesthesiol Scand.

may also be involved in spinal opioid pain inhibition since opioid antinociception is augumented by atropine[22]. Clonidine may also interfere with central cholinergic systems in at least three ways[23]. Firstly, clonidine might be agonistic at presynaptic α_2-receptors located on cholinergic neurons, resulting in an inhibition of acetylcholine release from cholinergic nerve terminals. Secondly, clonidine has been suggested to block muscarinic receptors. Both of these effects would decrease the physostigmine effect. Thirdly, clonidine is suggested to be an acetylcholinesterase inhibitor in itself, which would promote cholinergic antinociception. The net effect of clonidine interactions in spinal pathways for analgesia is at present not obvious[23].

Crosstolerance to analgesia in carbachol-tolerant rats
A study has been carried out to assess whether rats tolerant to the antinociceptive effects of spinal carbachol would demonstrate cross-tolerance to morphine (Fig. 3) and to guanfacine, a selective α_2-adrenoceptor agonist (Post, C. et al., unpublished). The spinal cords were also examined post-mortem by autoradiography for alterations of binding to cholinergic, noradrenergic and opioid receptors. Tolerance to the antinociceptive effects of spinal carbachol developed rapidly and was complete on the fourth day of injection. After intrathecal administration of guanfacine or morphine to carbachol tolerant rats an antinociceptive effect was measured, which was similar to that seen in drug-naive

animals. Autoradiography revealed a significant decrease of cholinergic receptor binding as visualized with [^{3}H]-methyl-QNB in the spinal dorsal horn of carbachol tolerant animals. Furthermore, downregulation of noradrenergic binding in the ventral horns and of opioid receptor binding in the dorsal horns was also observed.

Neurotoxicity to the spinal cord of cholinomimetic drugs
Morphologic studies of possible neurotoxic effects of cholinergic drugs on the spinal cord have been carried out following large intrathecal doses to rats daily for 14 days. The morphology of the dissected spinal cords was examined by light and electron microscopy and evaluated by morphometric methods. Chronic intrathecal administration of carbachol to rats produced no structural changes on the spinal cord (Gordh, T. et al., unpublished). Preliminary data showed that intrathecally administered carbachol produced an increased spinal cord blood flow. Additional information must be collected, however, before clinical studies with intrathecal injection of carbachol can be recommended.

Clinical studies
Analgesic effect of physostigmine in the early postoperative period
Studies in humans of the analgesic effect of cholinomimetic drugs have been almost purely experimental. However, it has been shown that both oral and parenteral administration of physostigmine and THA in selected patients rendered good analgesia and potentiated the effects of opioids[9,24-26]. Therefore, a double blind trial was pursued to assess the analgesic and antisedative effects of 2 mg i.v. physostigmine in surgical patients in the first postoperative hours[27]. Pethidine, 50 mg, and placebo were included for comparative purposes. The degree of pain and sedation was estimated when the patient demanded analgesia and immediately before administration of the test drug. In addition, ventilatory rate and side effects were monitored. The results showed that physostigmine caused analgesia that was of the same magnitude as pethidine during the first 15 min after which it decreased to the level of placebo (Fig. 3). An antagonistic effect of physostigmine on postoperative sedation was recorded over a somewhat longer time period. In contrast to pethidine, physostigmine caused no decrease in ventilatory rate. Although the duration of arousal and analgesic effects of physostigmine was short the use of physostigmine was suggested to be preferable to the use of, for example, naloxone when an immediate alertness of the patient is wanted without causing an increase in postoperative pain. The arousal effect of the combination physostigmine and naloxone postoperatively has also been confirmed in a large scale study[28].

The short duration of action of intravenous physostigmine was paralleled by a rapid plasma elimination[29]. An elimination half-life of about 22 min was estimated. An infusion dosage regimen of physostigmine was tried in postoperative patients in order to prolong the drug action in the postoperative patient.

However, the arousal effect was considered no better than after an intravenous bolus dose of physostigmine[30].

Cholinergic drugs in the treatment of severe neurogenic pain

Studies have shown that both subcutaneous and oral physostigmine produce pain relief in central neurogenic pain. An analgesic effect with a peripheral site of action has been suggested[26] since some patients with causalgia had good pain relief following administration of regional cholinesterase inhibitors and also after intravenous neostigmine which is believed not to penetrate the blood–brain barrier. Oral physostigmine has been tested with good results in selected patients. The oral bioavailability of physostigmine is 25% (Ref. 29).

Oral distigmine bromide was shown to produce significant analgesia in patients suffering from thalamic pain (Hampf, G., unpublished). Furthermore, addition of distigmine improved analgesia from amitriptyline in certain chronic pain states and decreased side effects.

9-Aminotetrahydroacridine, THA, has been used for the relief of pain and to potentiate morphine analgesia in intractable pain states[9]. The presumed advantage of THA over physostigmine is longer duration of action due to a slower plasma elimination. Recently a pharmacokinetic study in humans of THA was conducted[31]. As for other cholinesterase inhibitors the clearance was high with a resulting plasma elimination half-life in the range 70–200 min. The oral bioavailability was low with a mean of 17% (Ref. 31). THA effects were also studied in a group of postoperative patients, but the doses used did not result in any significant analgesia (Hartvig, P. et al., unpublished).

Conclusions

There are strong indications that muscarinic acetylcholine receptor mechanisms play a key role in pain management. Analgesia following the systemic administration of such cholinesterase inhibitors as physostigmine and THA has been demonstrated in postoperative pain and in cancer patients. Studies indicate an analgesic effect of physostigmine and distigmine in central neurogenic pain and in cases of causalgia. The spinal route of administration for muscarinic receptor agonists is of particular interest due to the good antinociceptive effect with long duration in the rat. Another interesting approach is the combination of agonists for different receptor types to potentiate the antinociception. Further experimental and clinical studies are necessary in order to increase our insight into the neuronal interactions behind spinal analgesia. It is obvious, however, that cholinergic mechanisms may play an important role in this complex scheme.

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Muscarinic receptor subtypes in human neurodegenerative disorders: focus on Alzheimer's disease

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Much evidence has clearly revealed the existence of marked cholinergic deficits in cortical and hippocampal areas in Alzheimer's disease. Although not necessarily of etiological origin, these deficits have been associated with learning and memory disabilities observed in this neurogenerative disorder. We report here that in addition to deficits in choline acetyltransferase (ChAT) activity, the maximal densities of high affinity [3H]acetylcholine and [3H]AF-DX 116 (possibly M_2), but not M_1 muscarinic receptor binding sites are decreased in cortex and hippocampus in Alzheimer's disease. Similar findings are also observed in Parkinson's disease with Alzheimer's type dementia. Additionally, animal studies suggest that a population of M_2 receptors is presynaptically located on cholinergic nerve terminals where they can act as negative autoreceptors to decrease acetylcholine release. Interestingly, blockade of these sites facilitates acetylcholine release and learning in rats. This may be relevant for the design of more appropriate therapeutic approaches toward the treatment of certain symptoms of Alzheimer's disease.

It has recently become evident that neurochemical deficits observed in Alzheimer's disease (AD) are varied and complex[1,2]. For example, alterations in various monoaminergic and neuropeptide systems have been reported[1,2]. However, it is clear that the most consistent findings are related to deficits observed in forebrain cholinergic and somatostatin neuronal markers. Other deficits are most likely seen only in advanced stages of the disease and/or in certain AD subtypes. Consequently, most studies have focused on the possible role of brain cholinergic neurons in the clinical features of AD.

Multiple neuropathological and neurochemical data have revealed that the nucleus basalis of Meynert (nbm)–cortical, and septo-hippocampal cholinergic pathways are most affected in AD (for example, see Refs 3 and 4). Since it is difficult to determine accurately acetylcholine (ACh) levels in post-mortem tissue, most studies have monitored the activity of the enzyme choline acetyltransferase (ChAT) as an index of cholinergic nerve activity[1–4]. Cholinergic receptors of the muscarinic and nicotinic subtypes have also been investigated as additional markers of the integrity of the cholinergic synapse in AD. While most studies have reported marked decreases in the densities of nicotinic receptors in cortical and hippocampal areas in AD brains[5–8], no clear consensus has been reached for muscarinic sites[2,8–13]. This may be related to the use of non-selective ligands, at a single concentration, making it difficult to differentiate between changes in affinities (K_d) and maximal number of sites (B_{max}). It is also likely that the existence of multiple brain muscarinic receptor

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subtypes may have been a confounding variable in this regard.

Recently, we began a detailed study of muscarinic receptor subtypes in various cortical and sub-cortical areas in AD brain tissues. Demented (Parkinson's disease with AD or PD/AD) and non-demented Parkinsonians were also studied for comparison. In all cases, complete saturation analyses were performed using selective ligands such as [3H]pirenzepine for the M_1 subtype[14] and [3H]ACh[8,15] and [3H]AF-DX 116[16] for the M_2 sites. The possible respective roles of these muscarinic receptor populations were also investigated using a variety of neurochemical and behavioral paradigms.

Cholinergic markers in various neurological disorders
ChAT activity

As previously reported, ChAT activity is markedly decreased in various cortical areas, hippocampus and nbm in AD brains[1–5] (Table I). In contrast, ChAT activity is not altered in the striatum and thalamus in this disease (Table I). A similar pattern is also observed in PD/AD brains, even though the decrements in ChAT activity are not as marked as in AD alone (Table I). This could be related to the relatively less advanced stage of the dementia of the AD type in the PD/AD patients (Robitaille *et al.*, unpublished).

Rather surprisingly, ChAT activity is also decreased in frontal and temporal cortices in nondemented PD brains (Table I). However, ChAT activity is normal in the striatum and thalamus (Table I). Similar results have recently been reported by others[17,18] suggesting that alterations in ChAT activity may precede clinically diagnosed dementia in these patients. Moreover, the relative preservation of hippocampal ChAT activity (Table I) may also be related to the apparent lack of dementia observed in these PD patients.

It is also of interest to note that alterations in ChAT

TABLE I Choline acetyltransferase activity (ChAT) in human brain

Area	ChAT activity (nmol ACh/mg protein/h)			
	control	AD	PD/AD	PD
Cortical				
Frontal cortex	6 6 ± 0 7	2 2 ± 0.3[a]	4 7 ± 0 9[c]	3 ± 0 9[b]
Temporal cortex	6 8 ± 0.9	1 9 ± 0 3[a]	4.2 ± 0 7[c]	3 2 ± 0 8[b]
Occipital cortex	2 9 ± 0 5	1.9 ± 0.2[c]	—	—
Hippocampus	14 1 ± 1 8	6 5 ± 1 0[b]	9 3 ± 2.2[c]	11.0 ± 2 5
Subcortical				
Striatum	38.4 ± 4 3	38 8 ± 4 1	43 3 ± 9 9	36 8 ± 7 9
Thalamus	18.5 ± 3.2	22.2 ± 3 0	14 0 ± 2.1	14.3 ± 1 5

All assays were performed in duplicate[8] Data are means ± s e values from six brains in each group for PD/AD and PD, and from 17 brains in each group for control and AD. Same brains were used for binding assays. [a]$p < 0.001$, [b]$p < 0.01$ and [c]$p < 0.05$ compared with controls

activity in cortical areas in AD brains are highly correlated to losses of nicotinic ACh receptor sites[8]. This suggests that nicotinic receptors are directly located on cholinergic nerve terminals in these brain regions. In fact, we have recently shown that these sites most likely function as 'positive' autoreceptors that insure the maintenance of constant basal levels of ACh in the synaptic environment[19].

M_1 muscarinic receptors

Using [^{3}H]pirenzepine as a relatively selective M_1 ligand, it was found that M_1 receptor binding parameters are generally unaltered (certainly not decreased) in AD brains[8]. As shown in Table II, slight increases in [^{3}H]pirenzepine binding are found in the hippocampus and striatum while binding is normal in all other areas. Similar results are obtained in PD/AD brain tissues, although the increase in [^{3}H]pirenzepine binding did not reach statistical significance in the hippocampus (Table II). In PD brains, we observed a significant decrease in the density of M_1 sites in the frontal cortex while binding in other regions was unaltered (Table II). Thus, it seems that the density of M_1 receptor sites is differentially altered in AD and PD brain tissues. Finally, it is also of interest to note that no correlation is found between changes in ChAT activity and M_1 receptor binding parameters in AD brains (Fig. 1).

M_2 muscarinic receptors

Due to the absence of highly selective ligands, it has been more difficult to characterize other brain muscarinic receptor sites. Thus, various radioligands and incubation conditions have been used in an attempt to selectively label non-M_1 sites, including [^{3}H]QNB or [^{3}H]N-methylscopolamine in the presence of M_1 blockers[10,14,20], [^{3}H]oxotremorine-M[14,21], [^{3}H]ACh[8,15] and more recently, [^{3}H]AF-DX 116[22-24].

On the basis of [^{3}H]ACh ligand selectivity pattern and receptor distribution[8,15,24], we chose to use this ligand as a probe for the determination of M_2 receptor binding parameters in human brain. As shown in Table III, the densities of [^{3}H]ACh sites are markedly decreased in various cortical areas and the hippocampus

TABLE II [^{3}H]Pirenzepine/M_1 binding parameters in human brain

Area	K_d (nM)				B_{max} (fmol/mg protein)			
	control	AD	PD/AD	PD	control	AD	PD/AD	PD
Cortical								
Frontal cortex	13 7 ± 1 8	16 2 ± 3.2	12.0 ± 1 1	10 0 ± 1.8	408 ± 59	366 ± 32	405 ± 60	242 ± 51[c]
Temporal cortex	12.6 ± 1.6	15 1 ± 2.8	9 6 ± 1.5	13 8 ± 4.9	365 ± 32	411 ± 53	596 ± 195	376 ± 60
Occipital cortex	19 6 ± 2.7	15.9 ± 2 3	—	—	419 ± 60	305 ± 51	—	—
Hippocampus	11 7 ± 2 8	17.6 ± 3 3	10.6 ± 1 7	10.5 ± 2.1	258 ± 39	363 ± 19[b]	333 ± 87	177 ± 51
Subcortical								
Striatum	17.8 ± 4.3	17.1 ± 3.5	12.2 ± 1 9	14.3 ± 4 8	466 ± 37	716 ± 57[a]	883 ± 147[b]	587 ± 85
Thalamus	34 6 ± 6 2	39.4 ± 6.1	—	—	443 ± 39	465 ± 37	—	—

Data are derived from complete saturation analysis Nonspecific binding in the presence of 1 0 μM atropine has been subtracted from all values[8] Results represent the maximal binding capacity (B_{max}) and apparent dissociation constant (K_d) for [^{3}H]pirenzepine binding and are expressed as the mean ± s e from six brains in each group for PD/AD and PD, and from 17 brains in each group for control and AD Same brains were used for ChAT and [^{3}H]ACh binding assays [a]$p < 0.001$, [b]$p < 0.01$ and [c]$p < 0.05$ compared with controls.
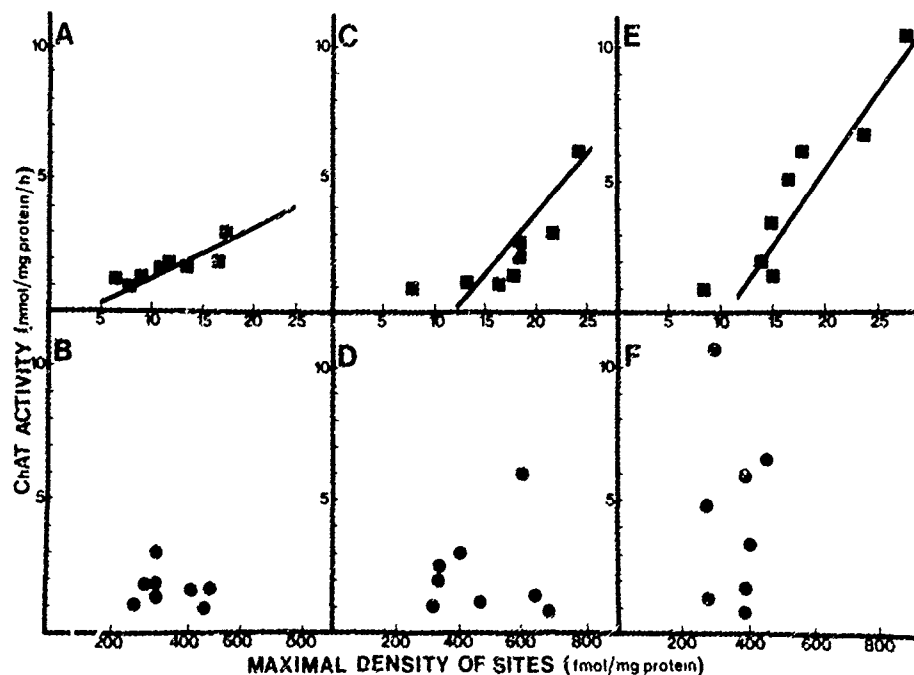
Fig 1. Relationship between ChAT activity and maximal density (B_{max}) of muscarinic receptor sites in Alzheimer's disease (AD) ChAT activity in the frontal cortex (A, B), temporal cortex (C, D) and hippocampus (B, F) of eight AD brains was measured and plotted against the B_{max} of [3H]acetylcholine (top row) and [3H]pirenzepine (M_1) (bottom row) binding in these areas The significance of the correlation between ChAT activity and the B_{max} of the ligand in each area was assessed using Pearson's correlative coefficient. Correlations between ChAT/[3H]ACh binding in frontal (r = 0.86, df = 6, p <0 01) and temporal (r = 0 82, df = 6, p <0.01) cortices, and hippocampus (r = 0 93, df = 6, p <0 001) were highly significant. No correlation was observed between ChAT/M_1 binding in frontal (r = −0.14, df = 6, p > 0 75) and temporal (r = 0 12, df = 6, p > 0.77) cortices and the hippocampus (r = −0 06, df = 6, p > 0.89). Modified from Araujo et al.[8] with permission

in AD. [3H]ACh binding in subcortical areas like the thalamus and the striatum is not modified (Table III). This confirms results reported earlier by Mash et al., who used other assay conditions[10]. Moreover, these authors had also suggested that this population of sites

might be located presynaptically, thus explaining their diminution in selected areas of AD brain tissues[10]. As shown in Fig. 1, we found a highly positive correlation between alterations in ChAT activity and [3H]ACh binding sites in frontal and temporal cortices, and in

TABLE III [3H]ACh binding parameters in human brain

Area	K_d (nM)				B_{max} (fmol/mg protein)			
	control	AD	PD/AD	PD	control	AD	PD/AD	PD
Cortical								
Frontal cortex	5.6 ± 0.8	7.3 ± 2.1	5 4 ± 3.3	3 7 ± 0 9	22.8 ± 1.4	11 6 ± 1 5[a]	8 3 ± 4 2[a]	6.8 ± 2.8[a]
Temporal cortex	6 6 ± 1.9	4.7 ± 1.6	5.0 ± 2.9	3.9 ± 1 6	30 3 ± 1.6	17 1 ± 1.8[a]	10 0 ± 5 2[b]	8 2 ± 2 0[b]
Occipital cortex	4.8 ± 1 0	5.4 ± 0.7	–	–	24.9 ± 1.4	16 8 ± 1 4[b]	–	–
Hippocampus	7.7 ± 2.4	8 8 ± 2 7	6 3 ± 2 4	4 3 ± 1 6	39.3 ± 3.0	17 1 ± 2 0[a]	–	13 0 ± 6 9[b]
Subcortical								
Striatum	8 6 ± 3 9	10.1 ± 4.7	2.7 ± 1.4[c]	7 1 ± 2 7	39 6 ± 3 3	42.3 ± 3.4	25 0 ± 11.2	14 3 ± 6 9[b]
Thalamus	7.6 ± 2.3	12 9 ± 4.0	–	8.5 ± 1.7	35 7 ± 3 1	32 4 ± 2 7	–	6.3 ± 1 1[a]

Data are derived from saturation analysis Nonspecific binding in the presence of 1 0 nM atropine has been subtracted from all values[a]. Results represent the maximal binding capacity (B_{max}) and apparent dissociation constant (K_d) for [3H]ACh binding and are expressed as the mean ± s.e. from three brains in each group for PD/AD and PD, and from eight brains in each group for control and AD. Additional brains are currently being analysed to match numbers used for ChAT and M_1 binding studies [a]p < 0 001, [b]p <0.01 and [c]p < 0.05 compared with controls

the hippocampus in AD brains. This further supports the hypothesis that [³H]ACh labelled sites are located presynaptically, since it is well known that the enzyme ChAT is present in cholinergic cell bodies and nerve terminals.

Recently, [³H]AF-DX 116 has been used as a more selective M_2 receptor probe[22-24]. Using this ligand, both Wang et al.[25] and we[26] have obtained data which are in agreement with those reported for [³H]ACh. Thus, it seems justified to conclude that the density of M_2 receptor sites is significantly decreased in various cortical areas and in the hippocampal formation in AD brains.

Similar decrements in [³H]ACh sites were also seen in cortical areas in PD and PD/AD brains[18] (Table III). As discussed for ChAT, this may seem rather surprising in the case of PD. On the other hand, it could be that decreases in presynaptic markers such as ChAT and [³H]ACh sites precede the appearance of clinical symptoms associated with dementia[18]. Further studies using brain tissues obtained at various stages of the disease should help to clarify this issue.

Finally, in contrast to AD, the densities of [³H]ACh (Table III) and [³H]AF-DX 116[26] binding sites in the striatum are decreased in PD and, most likely, in PD/AD brains. This is probably due to the alteration of the dopamine/ACh ratio which results from the massive loss of dopaminergic nerve terminals in the striatum observed in PD and PD/AD brains.

Possible functional roles of brain muscarinic receptor subtypes

The roles of the various brain muscarinic receptor subtypes still remain to be fully established. However, Potter et al.[14] have suggested that M_1 receptors may mediate excitatory events while M_2 sites may induce inhibition by regulating ACh release.

Using AF-DX 116 as a relatively selective M_2 receptor antagonist, we have recently characterized the differences between M_1 and M_2 muscarinic agents on ACh release[27]. As shown in Fig. 2A, AF-DX 116 and atropine stimulate, in a concentration-dependent manner, the potassium-evoked release of endogenous ACh from rat hippocampal slices. Similar effects are, although different in magnitude, also observed in frontal cortex and striatum[27]. The relatively selective M_1 receptor antagonist pirenzepine and the M_1/M_3 blocker 4-DAMP do not alter spontaneous or evoked ACh release (Fig. 2). Additionally, AF-DX 116 and atropine can reverse the oxotremorine-induced inhibition of evoked ACh release, while both pirenzepine and 4-DAMP are ineffective (Fig. 2B). Thus, these results suggest that 'negative' autoreceptors of the M_2 subtype can regulate ACh release from nerve terminals in the cortex, hippocampus and striatum in the rat brain.

More recently, we obtained evidence for the existence of functional M_2 autoreceptor sites, *in vivo*. Using a transcortical dialysis method, it was possible to demonstrate that acute infusions of muscarinic antagonists such as atropine and AF-DX 116, but not pirenzepine, into a dialysis probe, induced a long-lasting (4–5 h), marked release of ACh in non-anesthetized, freely moving rats[28]. Naturally, it will now be important to determine if it is possible to sustain stimulated release

following chronic blockade of the M_2 autoreceptor sites.

There is now abundant evidence suggesting that
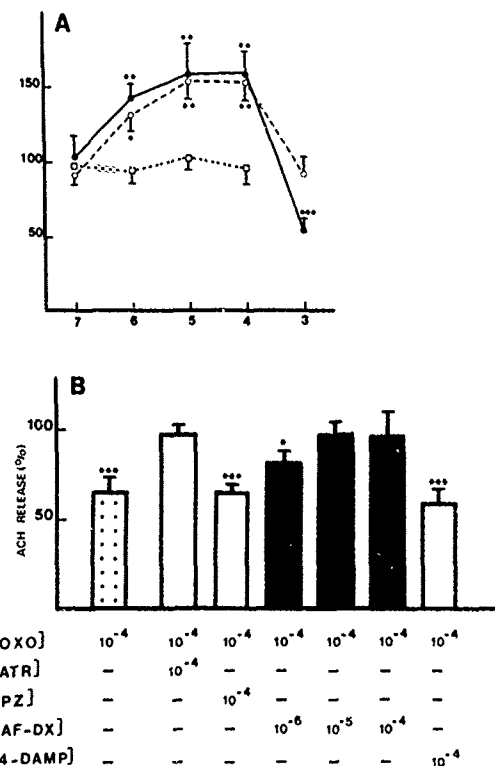
Fig 2. A Effects of muscarinic antagonists on the K⁺ (25 mM)-evoked release of acetylcholine from rat hippocampal slice preparations. Tissues were depolarized with Krebs medium containing 25 mM K⁺, in the presence or absence (control) of various concentrations of AF-DX 116 (●), atropine (○) or pirenzepine (■) Results are expressed as the mean ± s e of 5–7 experiments for each drug concentration. The overall control (no drugs) value (100%) for K⁺-evoked acetylcholine release was 7 9 ± 0 7 pmol/mg tissue/20 min in the hippocampus Statistical significance was assessed by Student's unpaired t-test by comparing each test value to its corresponding contralateral control (no drugs) Modified from Lapchack et al.[27], with permission ($^*p <0.05$; $^{**}p <0 01$, $^{***}p <0.001$)

B. Effects of muscarinic antagonists on the oxotremorine-induced inhibition of evoked acetylcholine release from rat hippocampal slice preparations Slices of tissues were incubated in the presence or absence (control) of oxotremorine (OXO, 10⁻⁴M), and one of the following: atropine (ATR, 10⁻⁴M), pirenzepine (PZ, 10⁻⁴M), AF-DX 116 (AF-DX, 10⁻⁶, 10⁻⁵, 10⁻⁴M) or 4-DAMP (10⁻⁴M) Results are expressed as mean ± s e of 5–6 experiments per drug concentration tested. The overall control value (100%) for acetylcholine release was 8.6 ± 0 5 pmol/mg tissue/20 min Statistical significance was assessed by Student's unpaired t-test by comparing each test value to its corresponding contralateral control (no drugs). Modified from Lapchak et al [27], with permission ($^*p <0.05$, $^{**}p <0 01$, $^{***}p <0 001$)

cholinergic neurons play a major role in the facilitation of learning and memory (for review, see Ref. 29). Consequently, one of the most important questions to answer is whether it may be possible to improve these behaviors by blocking M_2 inhibitory autoreceptors which should increase ACh levels in the synaptic cleft. It appears that this may be possible since in some learning paradigms we observed that post-training injections of AF-DX 116 in the range of 0.5–2.0 mg/kg (s.c.) result in a facilitation of memory in both win-shift and win-stay radial maze tasks in rats[30]. We also obtained evidence suggesting that AF-DX 116 can, at least partly, penetrate the brain following a peripheral injection[30].

Possible clinical significance

These results suggest that the selective antagonism of muscarinic M_2 autoreceptors m~~ result in the enhancement of ACh release *in vi* ~his could have therapeutic applications in rel~) learning and memory.

Muscarinic agonist (arecoline, bethanechol) therapies have been tested with relatively little success in AD patients. However, the data reported here suggest that major benefits should not be expected when using non-selective muscarinic receptor agonists. In fact, these agents would result in a decreased release of ACh from the remaining neurons in the brains of patients afflicted with AD, by acting at M_2 autoreceptor sites. Thus, the use of either M_1 selective agonists or M_2 autoreceptor antagonists may be more effective for the treatment of certain clinical features of AD or PD/AD. Alternatively, the use of a partial agonist/antagonist which acts presynaptically to enhance ACh release and postsynaptically to stimulate M_1 receptors could be most useful in this respect. Moreover, the combination of an M_2 autoreceptor antagonist and a cholinesterase blocker could be most effective in insuring the maintenance of high amounts of ACh at the synaptic level. This hypothesis certainly deserves further consideration.

Finally, the recent discovery of multiple muscarinic receptor genes[31] suggests that we are still far from having fully examined the possible usefulness of highly selective agents acting on a given muscarinic receptor subtype in various neurological disorders including AD, PD/AD and PD. The development of such compounds should prove to be helpful in establishing the respective roles of each muscarinic receptor subtype in the CNS.

Acknowledgements

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Muscarinic agonists for senile dementia: past experience and future trends

Julian A. Gray, Albert Enz and René Spiegel

Clinical experience with muscarinic agonists in the symptomatic treatment of Alzheimer's disease includes studies of the effects of pilocarpine, arecoline, bethanechol, oxotremorine and RS 86. Although the results are somewhat conflicting, there is evidence that a subgroup of patients may respond with an improvement of cognitive and/or behavioural function. The existing agents tend to induce adverse effects due to the stimulation of peripheral muscarinic receptors. Furthermore they reduce (at least in vitro) acetylcholine release by an action on presynaptic receptors. Strategies to overcome these problems include the development of potent agonists with high blood–brain barrier penetration, the search for agents selective for muscarinic receptor subtypes (using cloned receptors as tools) and the identification of agents acting as presynaptic receptor antagonists, to increase acetylcholine release.

The finding of a cholinergic deficit in the brain of patients with Alzheimer's disease has led to attempts at restoring cholinergic function by means of cholinomimetic drugs[1]. Such agents include acetylcholinesterase inhibitors, precursors of acetylcholine such as choline and lecithin, and muscarinic receptor agonists. It is the latter approach, using muscarinic agonists, which will be reviewed in this article.

It must be stated in advance that the results of such studies have yielded mixed and often disappointing results. In the second part of the article, the possible reasons for the unspectacular results achieved so far will be discussed, followed by a description of future trends to overcome these problems and optimise this approach.

Past experience

The agents to be described (see Fig. 1) are all muscarinic agonists with mixed activity at muscarinic subtypes.

Pilocarpine

Caine[2] reported on oral administration of pilocarpine to two patients with presumed Alzheimer's disease. No effects on learning and memory were detected after three weeks of administration in either patient. Lecithin was added to the treatment in one of the cases, but again no cognitive effects were seen after a further three weeks of combined pilocarpine/lecithin therapy. Similarly no effects on cognitive function were seen in two other patients with memory disturbance, one with Korsakoff's syndrome, and another with amnesia secondary to surgical removal of a third-ventricular colloid cyst.

Arecoline

Arecoline is an agonist at muscarinic receptors, although at high doses it may also have nicotinic effects. Christie et al[3] reported on the effect of intravenous infusion of 2 or 4 mg arecoline in seven patients with presenile dementia of the Alzheimer

CNS Clinical Research and CNS Preclinical Research, Sandoz Ltd, Basle, Switzerland.

type. A significant enhancement of performance in a picture recognition test was found after the 4 mg infusion in comparison with the effect of saline under double-blind conditions in the same patients. A similar trend was seen after the 2 mg infusion. The effect was in general small, although in two patients there was a clear-cut and reproducible response.

More recently Tariot et al[4] reported on the effect of two hour intravenous infusions of arecoline at rates of 1, 2, and 4 mg/hour, compared with placebo in a double-blind, randomized trial. In keeping with the results of Christie et al[3], a slight improvement in picture recognition performance was seen during the 2 mg/hour infusion, although this did not reach statistical significance and was not seen at the 4 mg/hour dose. No significant changes were seen in other tests of memory and learning. Interestingly, psychomotor activation and slightly improved affect were reliably observed at the 1 and 2 mg/hour infusion rates whereas increasing psychomotor retardation was observed at the 4 mg/hour rate.

Bethanechol

Several authors have reported on the effect of intracerebroventricular (i.c.v.) infusion of bethanechol, a long-established muscarinic agonist. The central route of administration has the advantage of minimizing peripheral cholinergic effects, against which must be weighed the surgical risks of implantation of an intracranial catheter.

Harbaugh et al[5] first reported encouraging results in four patients receiving i.c.v. infusion of bethanechol or saline under single-blind conditions at doses of 0.05 to 0.7 mg/day over eight months. There were subjective reports from relatives of decreased confusion, increased initiative and improved memory.

In contrast, in a double-blind placebo-controlled study of ten patients with biopsy-proven Alzheimer's disease, Penn et al[6] found no effects of bethanechol on cognitive function after low-dose infusion (0.35 mg/day) over 12 weeks, nor in a subsequent open-dose trial of up to 1.75 mg/day in eight of the ten patients. A subset of patients (three out of ten) showed evidence of improvement in behaviour and daily functioning with low-dose (0.35 mg/day) bethanechol, while at moderate
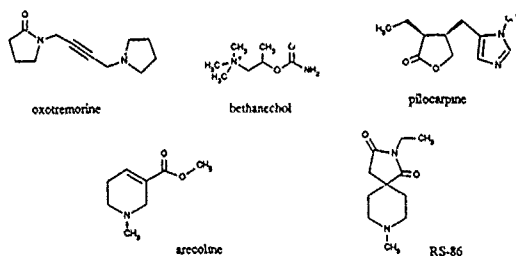
Fig 1 Muscarinic acetylcholine receptor agonists used in clinical trials

dose (1.05 mg/day) there appeared to be a more robust, significant improvement in mood, behaviour and activities of daily living. Higher doses, in contrast, were detrimental to behaviour.

There was a suggestion of decreased memory function at doses producing behavioural improvement, and the authors comment that it is not clear whether the degree of behavioural improvement with i.c.v. administration of bethanechol would be sufficient to justify the invasive neurosurgical procedure employed.

Finally, Read[7] reported on the effects in an open study of bethanechol infusion at individually titrated doses in 5 patients with probable Alzheimer's disease. Three of the patients were described as showing clinical improvement, with optimal doses of 0.20 mg, 0.35 and 0.95 mg per day respectively. Improvement in memory tests was paralleled by family reports of increased alertness and attentiveness at home. However, two patients developed agitation, irritability, depression and seizures, which were controlled with anticonvulsants, while a third patient suffered an intracerebral haematoma with focal seizures postoperatively, as well as a staphylococcal infection in the cerebrospinal fluid. The author suggests that individual titration may be highly important in order to obtain clinical benefit with i.c.v. bethanechol.

Oxotremorine

A double-blind study of oxotremorine (0.25 to 2.0 mg/day) orally versus placebo in seven patients with probable Alzheimer's disease resulted in depressive symptoms, often severe, in five out of the seven patients, making assessment of the drug's cognitive effects impossible[8].

The cholinergic hypothesis of affective illness is well known, and cholinomimetic drugs have previously been reported to produce depressive symptoms in normals and depressive patients[9]. However, in this context it is difficult to understand the *improvement* in mood reported after arecoline and bethanechol in some cases (see above).

RS 86

Perhaps the muscarinic agonist most studied in Alzheimer's disease in the last decade has been RS 86 (2-ethyl-8-methyl-2,8-diazospiro-4,5-decan-1,3-dione-hydrobromide; Sandoz Ltd.), a potent M_1 and M_2 receptor agonist with high brain penetration[10].

Five double-blind, placebo-controlled studies of repeated oral administration of RS 86 in patients with probable Alzheimer's disease have been performed Wettstein and Spiegel[11] reported on a crossover study over two 6-week periods with RS 86 (0.75 mg q.i.d.) and placebo in six patients with mild to severe dementia. Performance in a simple speeded visuomotor task was improved by 17 to 60 per cent in five of six patients on active drug and several, statistically non-significant trends in favour of RS 86 occurred in other psychometric tests. One patient displayed a clinically relevant improvement under active drug, becoming better orientated and showing less memory impairment in daily life, an effect confirmed by the marked deterioration on substituting placebo.

The same authors performed a further therapeutic trial in 17 patients with probable Alzheimer's disease of mild to moderate severity. A double-blind parallel-group period of six weeks was followed by two crossover periods of six weeks. A dosage of 1.0 mg t.i.d. RS 86 was compared with placebo, the dosage being reduced to 1.0 mg b.i.d. after 12 weeks due to a high dropout rate. Only five patients completed the 18-week period, six dropouts occurring on RS 86 and six on placebo. Dropouts on RS 86 treatment were due to nausea ($n = 1$), dizziness ($n = 1$), angina pectoris ($n = 2$), malaise ($n = 1$) and bronchospasm in one asthmatic patient. Sweating and increased salivation were commonly observed. The high number of dropouts prevented detailed statistical analysis. However, nine out of eleven cognitive and behavioural parameters showed higher median changes from baseline on RS 86 compared with placebo treatment. Two patients were considered by the attending nurses and physician to have shown improvement on RS 86 that was relevant in daily life.

Bruno et al.[12], however, reported essentially negative results with RS 86 in a double-blind, placebo-controlled crossover study over eight days in eight patients with clinically diagnosed Alzheimer's disease. RS 86 was administered at a dose of 0.5 mg daily, increased until either a dose of 5.0 mg per day was achieved or significant adverse effects developed. No statistically significant changes occurred in tests of cognitive function or attention. All patients reported sweating on RS 86, while hypersalivation occurred in two patients. The same authors[13] also reported negative results in seven patients with clinically diagnosed Alzheimer's disease receiving RS 86 at high dosage i.e. 10 mg per day, in combination with the peripherally active anticholinergic drug glycopyrrolate. The patients received increasing doses of RS 86, or placebo, in a double-blind, placebo-controlled crossover design. The maximum dose was reached after eight to ten days. Although several verbal and nonverbal tests yielded slightly higher scores during RS 86 treatment, there were no statistically significant or clinically relevant changes.

Finally, Hollander et al.[14] reported on their experience with RS 86 in twelve patients with probable Alzheimer's disease of mild to moderate severity. The patients first received, in an open dose-finding study, placebo followed by 0.5 mg, 1.0 mg and 1.5 mg of RS 86 orally three times daily for seven days at each dose. The 'best dose' as assessed by effects on the Alzheimer's

Disease Assessment Scale (ADAS), was then used in a double-blind, placebo-controlled crossover study of two weeks' duration for each phase. Three patients dropped out of the study because of adverse events occurring while taking RS 86, namely syncope, abdominal distress, and a seizure. Seven out of twelve patients showed improvement on the overall ADAS scores in the replication phase, with improvements ranging from 3.4 to 36.2 per cent. In two cases the improvement was clinically obvious, being commented on spontaneously by family and staff, yet subscale analysis did not reveal any predominant effect on cognitive function as compared with non-cognitive behaviour.

In summary the results to date suggest that a minority of patients with Alzheimer's disease may show a clinically relevant response to existing muscarinic agonists. It has not been possible to identify responders in all studies according to clinical criteria, although Hollander *et al* [14] suggested that less severely impaired patients were more likely to improve on cognitive testing.

Possible explanations for this low responder rate include both general problems with the 'cholinergic substitution' approach and specific problems of existing muscarinic agonists. Possible general criticisms of the use of muscarinic agonists as monotherapy in Alzheimer's disease include: (1) that there exist additional non-cholinergic central neurotransmitter losses in Alzheimer's disease, so that muscarinic receptor stimulation alone may be insufficient to induce a clinical response; and (2) that tonic stimulation of muscarinic receptors may not be sufficient to replace 'phasic' physiological stimulation.

In spite of these objections further pursuit of the muscarinic agonist approach to treatment seems justified since, in a severe degenerative condition such as Alzheimer's disease, effective symptomatic treatment even in a minority of cases would be a significant medical advance. Furthermore, in order rigorously to test the hypothesis that muscarinic agonists may be effective, it will be necessary to develop better-tolerated and more sophisticated compounds, as will now be described.

Future trends

Peripheral cholinergic side-effects remain a source of concern with non-selective muscarinic agonists, often limiting the clinical dose range. One possible solution to this problem is the development of highly lipid-soluble, potent muscarinic agonists, which will penetrate the blood–brain barrier readily and lead to central muscarinic activation at relatively low plasma levels which are associated with little peripheral activity.

This approach is borne out in laboratory and human pharmacological experiments with SDZ BOP 086 [(−)-s-2,8-dimethyl-1,3-dioxa-8-azaspiro-[4,5]-decane hydrochloride (Sandoz Ltd.)], a potent M_1 and M_2 receptor agonist according to the classical pharmacological nomenclature. Central cholinergic activation is achieved in humans with this agent, as evidenced by shortening of REM latency in the sleep EEG in male volunteers at doses (0.5 mg and 1.0 mg) below those at which peripheral cholinergic side-effects are reported. Unfortunately, clinical development of this compound has had to be discontinued owing to hepatotoxicity.

A second theoretical approach to the development of better-tolerated muscarinic agonists is the search for agonists selective for the muscarinic receptor.

Unfortunately the search for M_1-selective agonists has proved a difficult task for chemists to date. The situation may change, however, with advances in our knowledge concerning muscarinic receptor subtypes.

Recently, through the advances in molecular biology techniques, cDNAs and genes for five muscarinic subtypes have been cloned and expressed in cell lines devoid of endogenous receptors[15,16]. Studies using these cloned receptors provide a new basis for characterization of the function and distribution of muscarinic receptor subtypes and a new approach to the search for selective muscarinic agonists. Interestingly, the cloned muscarinic receptors share partially the biochemical function of the pharmacologically defined receptors (see Fig. 2).

In addition to postsynaptic receptor stimulation, muscarinic receptor agonists also tend to reduce, at least *in vitro*, the release of acetylcholine through stimulation of presynaptic autoreceptors, which have a low affinity for pirenzepine[17]. Such an action *in vivo* might counteract the positive effects of postsynaptic activation and reduce the therapeutic usefulness of currently available muscarinic agonists, which generally possess mixed activity at several receptor subtypes.

A novel approach to this problem is the development of specific presynaptic muscarinic receptor antagonists.

Cloned receptor subtype	m 1		m 2		m 3		m 4		m 5	
Pharmacological equivalent	M_1 neuronal		M_2 cardiac		M_3 glandular		M ?		M ?	
	PI	cAMP	PI	cAMP	PI	cAMP	PI	cAMP	PI	cAMP
Influence on second messenger systems	↑	↑	↓	↓	↑	↑	↑	↓	↑	↑
Affinity for pirenzepine	+ + + +		+		+ +		+ + +		+ +	
Electrophysiological response*	opens		no effect		opens		no effect		?	

Fig. 2. Muscarinic acetylcholine receptor subtypes ↑, stimulation of inositol phosphate metabolism or adenylate cyclase; ↓, inhibition of adenylate cyclase. * Ca^{2+}-dependent effect on K^+ channels, ++++, high, and +, low affinity for pirenzepine.

The prov' ,p(substance of this class is BM-5 (Ref. 18). Perhaps ar 'de il compound would act both as a selective f re v lap.ic antagonist, liberating acetylcholine, and as a selective M_1 subtype-specific postsynaptic agonist.

In summary better understanding of cholinergic receptor pharmacology combined with advances in molecular biology techniques bodes well for the future in terms of development of muscarinic agonists suitable for clinical use. Despite the equivocal clinical data achieved in Alzheimer's disease with existing muscarinic agonists, a fair trial of the usefulness of muscarinic receptor stimulation must await the development of more suitable agents.

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Muscarinic pharmacology of the airways

Jennifer Maclagan and Peter J. Barnes*

Muscarinic receptors have been identified in the airways in several species, including humans, located on airway smooth muscle, secreting cells and on the nerves. M_1 receptors are found in sympathetic ganglia in the guinea-pig and in parasympathetic ganglia in humans. M_2 receptors (inhibitory autoreceptors) are found in cholinergic parasympathetic nerve terminals in many species, including humans, whereas the muscarinic receptors found on airway smooth muscle and mucus glands belong to the M_3 subtype. It is possible that a defect in neuronal M_2 receptor function may explain β-blocker-induced asthma. M_2 antagonists such as methoctramine are promising tools for elucidating the role of muscarinic receptor subtypes in the lung. However, they can potentially increase acetylcholine release. This property is not shown by drugs with a higher selectivity for M_1 and M_3 receptors which are likely to be useful clinically in the treatment of airway disease.

It has been known for many years that parasympathetic cholinergic nerves are the predominant neural pathways in the airways and cholinergic mechanisms may be important in airway obstruction in humans. Cholinergic nerves pass down the vagus nerve to synapse in ganglia within the airway walls from which short post-ganglionic fibres pass to the target cells, including airway smooth muscle and mucus glands. Release of acetylcholine (ACh) from the post-ganglionic nerve terminals onto postjunctional cholinoceptors leads to a contraction of airway smooth muscle and to mucus secretion; these effects are blocked by muscarinic antagonists such as atropine and ipratropium bromide.

Autoradiographic mapping studies have shown that muscarinic receptors are present on airway smooth muscle cells and on gland cells in the airways of animals[1] and humans[2], and the smooth muscle receptors from large to small airways appear to be entirely of the M_3 subtype. The classification of the postjunctional receptors into the M_3 category is supported by evidence from autoradiographic, ligand binding and pharmacological studies *in vivo* and *in vitro* obtained in many laboratories. Recent work has shown the existence of muscarinic receptors in the nerves supplying the lung in animals[3] and humans[4]. This has led to the concept that the neural control of airway smooth muscle is much more complex than had been originally envisaged. Classification of these neural receptors may have great relevance for future drug design for airway disease.

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M₁ receptors

M_1 receptors with a high affinity for pirenzepine are generally found in the cerebral cortex and on autonomic ganglia[5]. Pirenzepine is used clinically to reduce gastric acid secretion and although it was originally believed to act directly on the acid secreting cells, it is now apparent that it acts predominantly on parasympathetic ganglia in the stomach to inhibit neurally mediated gastric secretion. As the innervation of the airways is derived embryologically from the same origin as that of the gut, excitatory M_1 receptors seem likely to exist also in airway ganglia and they have now been identified. There appear, however, to be considerable species differences in the location of M_1 receptors in pulmonary autonomic nerves (see Fig. 1). Pirenzepine was shown to be very effective in blocking bronchoconstriction due to preganglionic stimulation of the vagus nerves in both rabbits[6] and dogs[7] and these results were interpreted as showing M_1 receptors in the pulmonary parasympathetic ganglia in these species. However, these studies did not clearly show that pirenzepine was effective at doses that had little action on the bronchoconstrictor effect of acetylcholine mediated via the postjunctional M_3 receptors.

Recent evidence suggests that M_1 receptors may be present on human airway cholinergic nerves. The effects of inhaled pirenzepine and ipratropium bromide on cholinergic reflex bronchoconstriction, triggered by inhalation of sulphur dioxide, were compared in atopic subjects (Fig. 2). A dose of inhaled pirenzepine was found that failed to inhibit significantly the bronchoconstriction due to inhaled methacholine (which acts directly on airway smooth muscle M_3 receptors), whereas ipratropium bromide was able to block this response as expected. The same dose of pirenzepine, however, was as effective as ipratropium bromide in blocking bronchoconstriction induced by sulphur dioxide, and as it could not be acting directly on airway smooth muscle receptors, it must be acting on some part of the cholinergic reflex pathway[8]. The parasympathetic ganglia seem to be the most likely location for this action, and human airway ganglia show a high density of muscarinic receptors in autoradiographic studies[2]. M_1 receptors have also been demonstrated in the parasympathetic ganglia of rabbit bronchi[9].

In the guinea-pig, M_1 receptors have also been demonstrated on pulmonary autonomic nerves but they appear to be located in sympathetic rather than in the parasympathetic ganglia. In this species, pirenzepine facilitated vagally-induced bronchoconstriction at doses which had no effect on airway smooth muscle muscarinic receptors. This facilitation was prevented by propranolol, suggesting that M_1 receptors may be localized in the sympathetic nervous pathway, where they exert a facilitatory role[10]. As sympathetic stimulation in the guinea-pig causes bronchodilation and also inhibits cholinergic neurotransmission, pirenzepine, by blocking sympathetic M_1 receptors, would reduce noradrenaline release and thus indirectly increase the effect of vagal nerve stimulation. These M_1 receptors may be in sympathetic ganglia or on the sympathetic nerve terminals which are closely associated with airway cholinergic nerves and ganglia (Fig. 1). Compar-
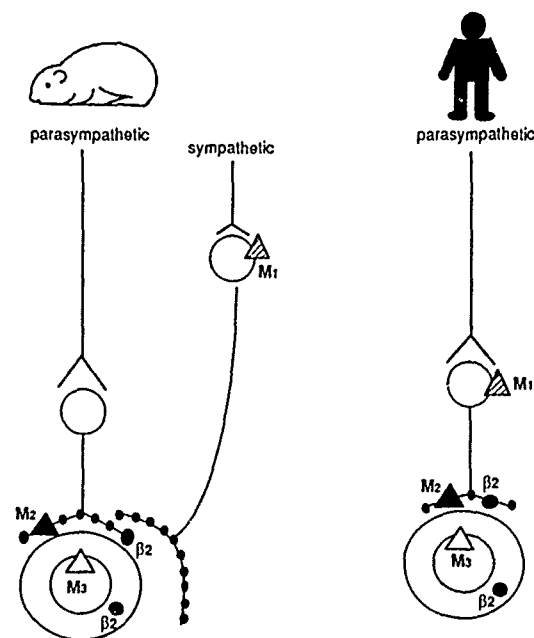
Fig 1 Location of muscarinic receptor subtypes in pulmonary autonomic nerves in guinea-pig and humans. M_1 (pirenzepine-sensitive) facilitatory receptors are localized to sympathetic nerves in the guinea-pig and to parasympathetic ganglia in humans M_2 inhibitory receptors are localized to post-ganglionic cholinergic nerves, which also have β_2-adrenoceptors, whereas M_3 receptors are present on airway smooth muscle in both species (The ganglionic acetylcholine nicotinic receptor has been omitted)

able mechanisms have not yet been investigated in human airways.

The physiological role of M_1 receptors in autonomic ganglia is still uncertain. Classically, ganglionic transmission is via nicotinic cholinoceptors which are activated by acetylcholine and nicotine and blocked by hexamethonium. Possibly excitatory M_1 receptors exert a facilitatory effect on neurotransmission and may thus amplify ganglionic transmission. Activation of these receptors probably closes potassium channels, resulting in a slow depolarization of the ganglion cell[11]. The M_1 receptors would therefore be expected to have a role in long-term regulation of cholinergic tone, whereas the nicotinic receptors are more important in rapid signalling. If so, M_1 antagonists such as pirenzepine or telenzepine might have a useful therapeutic role in asthma, as they may reduce vagal tone in humans. This may be especially important in nocturnal exacerbations of asthma, which may be associated with increased vagal tone[12].

M_1 receptors have been detected in the lungs by radioligand binding studies but the results are unexpected. Binding of [^{3}H]quinuclidinyl benzilate (QNB) to both rabbit and human peripheral lung membranes is displaced by pirenzepine with a shallow inhibitory curve suggesting the presence of high and low affinity

sites[13,14]. The high affinity binding site has the characteri.tics of an M_1 receptor and this is confirmed by the use of [³H]pirenzepine, which selectively labels this receptor subtype. M_1 receptors make up more than half the binding sites in human lung. This cannot possibly be accounted for by receptors on parasympathetic ganglia or nerves, which would make up only a small fraction of the membranes. Recent autoradiographic mapping has shown that the M_1 receptors are present on submucus glands but not on airway smooth muscle[15]. They are also found on the alveolar walls, which would account for the high density of M_1 receptors in peripheral lung, where the cholinergic innervation is very sparse and where the smooth muscle is lacking. However, the function of these M_1 receptors in human peripheral lung is unknown.

M_2 receptors

Muscarinic receptors have been described in the airways localized to the parasympathetic nerve terminals where they exert an inhibitory control of acetylcholine release. These receptors were first demon-

a

b

IB=ipratropium bromide, PZ=pirenzipine

Fig. 2 (a) Localization of the effect of sulphur dioxide (SO₂) to cause vagally-mediated bronchoconstriction compared to the direct effect of methacholine (MeCh) on airway smooth muscle. (b) Effect of pirenzepine (PZ) and ipratropium (IB) on bronchoconstriction induced by inhaled SO₂ and MeCh in atopic subjects (n = 7). Airway function was measured as a change in total lung resistance (Rrs) using a forced oscillation technique and a dose of MeCh which evoked a 40% increase in Rrs (PC₄₀). Single asterisk indicates p <0.05, double asterisks indicate p <0.02, mean values ± s e m Both inhaled PZ (70 µg and IB (7µg) significantly inhibited vagally-mediated bronchoconstriction elicited by SO₂ to the same extent However, at this dose, PZ had no effect on MeCh-induced bronchoconstriction, whereas IB gave significant protection This suggests that pirenzepine sensitive (M₁) receptors exist in the vagal reflex pathway, presumably located in parasympathetic ganglia (Adapted from Ref. 8.)

strated in guinea-pig lung using the antagonist gallamine[3] and have now been identified also in cat[16], dog[17] and human airways[4]. Blockade of these receptors with a selective antagonist causes facilitation of bronchoconstriction induced by vagal nerve stimulation[3,16]. As these receptors are activated by the transmitter at physiological rates of firing in the nerve, they function as autoreceptors. It has since been confirmed that these receptors are selectively blocked by methoctramine[18] and AF-DX 116, confirming their classification as M_2 receptors comparable to the M_2 receptors on the atrial cell which mediate slowing of the heart. The cardiac M_2 receptors are known to be coupled to K^+ channels and their activation causes K^+ efflux with resulting hyperpolarization of the cardiac cell (the opposite effect on K^+ efflux to that produced by M_1 facilitatory receptors). If the pulmonary neuronal M_2 receptors are also coupled to K^+ channels this could explain the inhibitory effect which M_2 receptors exert on acetylcholine release. This suggestion is supported by the finding that cromakalim, a K^+ channel opening drug, also causes inhibition of vagally-induced bronchoconstriction[19].

As these prejunctional M_2 receptors have a post-ganglionic location, they can be activated using field stimulation of in vitro preparations of the airways. Thus pilocarpine, an agonist for the prejunctional receptors, inhibited cholinergic nerve-induced contraction in guinea-pig[4,20] and human bronchi[4] elicited by field stimulation while having no effect on contraction induced by acetylcholine added to the bath. The suppressive effect of pilocarpine was blocked by gallamine, confirming that it was mediated via M_2 receptors.

In normal subjects, pilocarpine (in a dose which had a small bronchoconstrictor effect in its own right) was shown to have an inhibitory effect on cholinergic reflex bronchoconstriction induced by sulphur dioxide, whereas a similar degree of bronchoconstriction caused by histamine did not affect SO₂-induced bronchoconstriction[21]. This suggests that these inhibitory receptors may be present in normal human airways in vivo, presumably controlling cholinergic bronchoconstriction. In asthmatic patients, pilocarpine had no such inhibitory effect, suggesting that there might be some dysfunction of the autoreceptors, resulting in exaggerated cholinergic reflex responses.

M_3 receptors

Many laboratories have shown, using in vivo and in vitro methods, that the muscarinic receptors on airway smooth muscle are sensitive to antagonists such as 4-DAMP and hexahydro-sila-difenidol and are therefore classified as M_3 receptors comparable to the receptors in the ileum. Binding studies in guinea-pig lung membranes indicate a preponderance of M_3 receptors, whereas in human lung, M_3 receptors make up less than half the receptors (the remainder being M_1)[14]. In humans, autoradiographic mapping studies have confirmed that M_3 receptors are present on airway smooth muscle and submucus glands. These receptors seem to be linked to phosphoinositide breakdown with the production of inositol-1,4,5-trisphosphate which releases calcium ions from intracellular sites[22].

GUINEA-PIG
n = 5

Fig. 3 Summary of results comparing the effect of ipratropium on bronchoconstriction (measured as an increase in pulmonary inflation pressure measured from a side arm on the tracheal cannula, P_{pi}) and bradycardia These responses were induced either by vagal nerve stimulation (V.S, solid line and hatched columns) or by intravenous ACh (1 µg/kg, dashed line and open columns) The responses before ipratropium (B₁) are shown on the left 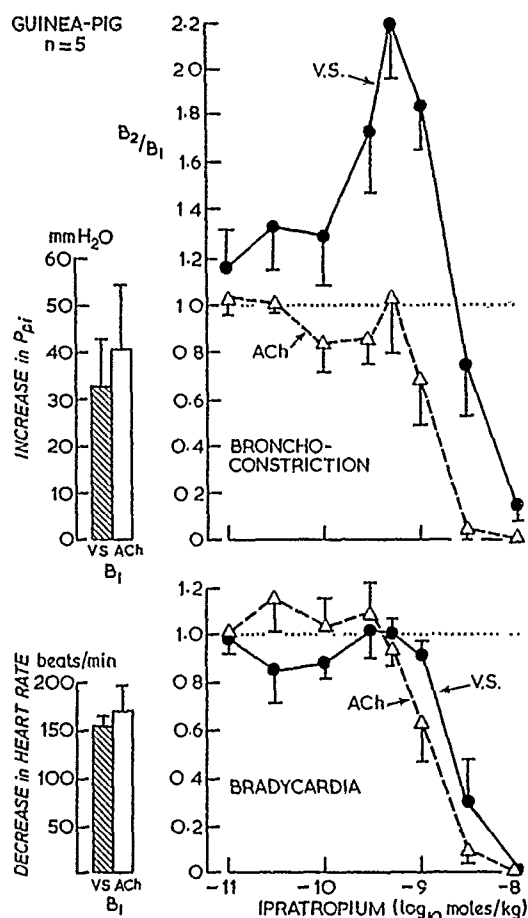in absolute units Results are expressed as the ratio B₂/B₁, where B₂ is the response after ipratropium (All points are the mean ± s.e., n = 5) Ipratropium initially enhances cholinergic nerve-induced bronchoconstriction, presumably by preventing feedback inhibition of acetylcholine release via M₂ receptors. As the dose is increased, blockade of the postjunctional receptors abolishes all pulmonary and cardiac responses (From Ref. 24)

The submucous glands appear to contain M₁ and M₃ receptors[15], both of which stimulate mucus secretion. Functional studies with selective antagonists suggest a response intermediate between M₁ and M₃ receptors, supporting the view that both types of receptor may play a part in airway mucus secretion[23].

Therapeutic implications

The discovery of three muscarinic receptor subtypes in the lung has important clinical implications and raises the possibility of producing more selective anticholinergic therapeutic agents in the future. The antimuscarinic bronchodilators currently available, atropine, ipratropium bromide and oxitropium bromide, are nonselective antagonists which block the neuronal receptors as well as the smooth muscle receptors. Ipratropium bromide has been shown in animals, at low doses, to increase vagally-mediated bronchoconstriction[24] due to blockade of the prejunctional M₂ receptors, as shown in Fig. 3. At this dose the responses of the postjunctional receptors to injected acetylcholine were unaltered. Abolition of all cholinergically mediated responses only occurred at higher doses when postjunctional M₃ receptor blockade was achieved. A similar mechanism may occur in patients and could explain some of the cases of paradoxical bronchoconstriction which have been reported with inhaled anticholinergic drugs in patients with chronic obstructive airway disease.

β-Blocker-induced asthma

The worsening of asthma by β-blocking drugs remains a problem and deaths continue to be reported. Even β-blocker eye drops are capable of precipitating an asthma attack. The cause has remained elusive, although cholinergic mechanisms may have a critical role, as anticholinergic drugs may completely prevent the bronchoconstriction due to inhaled propranolol in mild asthmatics[25]. Muscarinic autoreceptors might help to explain how catastrophic bronchoconstriction may be precipitated by even a small dose of a β-blocking drug. β-Blocking drugs inhibit sympathetic bronchodilator tone, which in humans is probably provided by circulating adrenaline. β₂-Adrenoceptors which, when activated, inhibit the release of acetylcholine, have now been demonstrated on cholinergic nerves of human bronchi[26], and when these are blocked an increase in tonic acetylcholine release would be expected. In normal individuals the acetylcholine would activate the autoreceptors to shut off any further acetylcholine release and thus no bronchoconstriction would occur. In asthmatic patients the same increase in acetylcholine would occur but, because of the apparent defect in muscarinic autoreceptors[21], there would be no mechanism for switching off acetylcholine release. Furthermore, the asthmatic airway is hyperresponsive to acetylcholine, thus even low doses of a β-blocker may precipitate a catastrophic bronchoconstrictor response[27].

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Selective antagonists reveal different functions of M cholinoceptor subtypes in humans

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Effects of atropine and of the subtype selective mAChR antagonists pirenzepine (PZ) and AF-DX 116 were studied in humans. Dose- or time–response curves were established for heart rate and salivary flow. Plasma samples were drawn in parallel with the effect measurements and analysed for drug concentrations. Subtype-selective radioreceptor assays of the samples served to estimate the respective receptor occupancy in vivo. It is shown that low doses of PZ (M_1-selective blockade) cause cholinomimetic effects indicated by bradycardia and increase in salivary flow. After high doses of PZ or atropine, tachycardia and inhibition of salivary flow are observed in parallel with occupancy of both the M_2 and M_3 subtypes. AF-DX 116 induces a tachycardia together with an increased salivary flow in agreement with its selectivity profile ($M_2 > M_1 > M_3$). The diagnostic and therapeutic applications of M_1- or M_2-selective blockade by low dose PZ or AF-DX 116 respectively are discussed.

Methods and tools borrowed from receptor pharmacology should allow for more detailed studies of the physiology and pathology of mAChR subtype driven processes in humans. Based on this better understanding, the wide variety of novel, subtype-selective ligands might be translated into useful diagnostic and therapeutic drugs. In our studies we have combined *in vitro* and clinical studies of antagonists to move towards this goal.

Combined *in vivo* and *in vitro* approach
In the present context, we have chosen two primary

parameters to quantitate mAChR effects in humans, i.e. heart rate and salivary flow. Only minor emphasis is put onto other mAChR-related parameters like intracardiac conductivity, respiratory variability of sinus node rhythm, blood pressure or accommodation capacity of the eye. As drugs we used atropine, the M_1-selective antagonist pirenzepine (PZ)[1] and the M_2-selective AF-DX 116, which differentiates between the cardiac (M_2) and glandular (M_3) subtype[2]. Dose–response of atropine or PZ and time–response curves of AF-DX 116 were established in healthy volunteers.

To link the respective response to the occupancy of a particular receptor subtype, we applied a technique used in similar studies of selective β-adrenoceptor blockade[3]: plasma samples were drawn in parallel with effect measurement and subjected to mAChR-subtype-selective radioreceptor assays (RRA). In these receptor binding assays we used [^{3}H]-PZ as a label for bovine cerebral cortex M_1 subtypes[4,5] and [^{3}H]-N-methyl-sco-

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polamine for the porcine atrium M_2 subtype[6] and rat salivary gland M_3 subtype[4,5]. From the inhibition of receptor binding by antagonist present in the plasma samples, occupancy of the respective receptor subtype and apparent plasma concentrations were calculated as described[3]. In preliminary experiments it was established that receptor binding of the radioligands was not affected by inclusion of up to two-thirds of native human plasma in the assay[4]. Thus, the assay of plasma samples was carried out without significantly perturbing the *in vivo* established equilibrium between free and bound drug. As shown earlier for β-blockers[3] as well as atropine and pirenzepine[4], the free drug concentration in the plasma compartment measured with these precautions is representative of the active drug concentration at the respective receptors oriented towards the extracellular space. Thus, we were in a position to detect *in vivo* receptor-occupying, active antagonist in the *in vitro* assay. Furthermore, we compared the affinity profiles of the antagonists used in the present study in different species including human tissues (Table I). In these receptor binding assays a twofold or less difference in the K_i values of the drugs for M_2 and M_3 receptors is apparent and we therefore feel safe in estimating the *in vivo* receptor subtype occupancy in humans from this RRA of plasma samples.

Peripheral M_1 receptor blockade generates M-cholinergic effects in humans

Cumulative dose–response curves of atropine and PZ were established without and after β-blockade by propranolol. As can be seen from Fig. 1 (panels B and C), PZ and atropine show biphasic dose–response curves for salivary flow and heart rate. This pattern is far more pronounced for pirenzepine than for atropine. ED50 values calculated from a model superimposing two dose–response curves with opposite effect vectors[4] show a 40- to 60-fold selectivity for the heart rate decelerating versus the accelerating effect of pirenzepine[5]. The respective parameters for atropine show

only a two- to four-fold selectivity[4]. The M, receptor subtype occupancy derived from the receptor assay of plasma samples (Fig. 1A) coincides with the *'paradoxical'* cardiodeceleratory and hypersalivatory effects of PZ. The *'typical'* antimuscarinic effects of PZ on salivary flow as well as heart rate are reflected in the occupancy of salivary M cholinoceptors. The preferential affinity of PZ for M_1 receptors also becomes apparent in the experiment depicted in Fig. 1D. From the above studies we selected a dose of PZ (1.1 mg), sufficient to block M_1 receptors in humans without a blockade of other subtypes. When the dose–response curve of atropine was established after pretreatment with this dose of PZ, only a monophasic response was observed (Fig. 1D). Thus, we can attribute the cardiodeceleratory effects of both atropine and pirenzepine to the same M_1 subtype. It should be pointed out that β-blockade was not able to abolish the cardiodeceleratory effect of the M_1-blockade (compare Fig. 1C and 1D, i.e. without and with β-blockade respectively).

To elucidate the mechanism underlying this observation, we studied postsynaptic vagal activity under the different conditions As a non-invasive measure we used the respiration induced variability of the sinus rhythm read from the RR-intervals of the ECG[7]. Increased postsynaptic activity has been reported to coincide with increased variability in the RR-intervals and vice versa. In our investigations the M_1-selective dose of PZ increased the variability of the RR-intervals by 58% above control. The maximum dose of atropine reduced the variability almost completely by 85% of control independent of pretreatment with PZ[4]. β-blockade did not affect these results.

From the above data we concluded that an M_1-selective blockade increases the postsynaptic M-cholinergic activity and we tried to narrow down the possible mechanism underlying this finding. Blockade of excitatory M_1 receptors located on peripheral sympathetic ganglia[8] would be a possible explanation for the cardiodeceleratory effects of low dose PZ[9].

TABLE I. K_i values of mAChR antagonists in different species

M-AChR antagonist	Atrium M_2 receptor		Salivary gland M_3 receptor			Cerebral cortex M_1 receptor
	porcine	human	rat	bovine	human	bovine
Atropine	3.4	4.7	1.9	2 4	3 8	0 8
	$n = 13$	$n = 3$	$n = 7$	$n = 5$	$n = 4$	$n = 13$
	SEM $= 0.5$	SEM $= 0.5$	SEM $= 0.4$	SEM $= 0.6$	SEM $= 0.4$	SEM $= 0.1$
Pirenzepine	1500	2320	533	928	1049	8.9
	$n = 6$	$n = 2$	$n = 6$	$n = 4$	$n = 3$	$n = 13$
	SEM $= 227$	SEM $= 127$	SEM $= 108$	SEM $= 20$	SEM $= 135$	SEM $= 0.8$
AF-DX 116	194	172	5150	6470	6180	644
	$n = 10$	$n = 3$	$n = 10$	$n = 5$	$n = 4$	$n = 8$
	SEM $= 21$	SEM $= 21$	SEM $= 1030$	SEM $= 407$	SEM $= 349$	SEM $= 160$

K_i values (nM) of atropine, PZ and AF-DX 116 in different tissues from different species. Human tissue samples were derived from surgical procedures, intact animal organs from the local slaughter house or from our animal facilities. Membranes were prepared from these materials after homogenization and several centrifugation and wash steps (see Ref 4). From receptor binding isotherms of the different drugs versus [3H]-PZ (M_1) or [3H]-N-methyl-scopolamine (M_2 and M_3) in filtration assays, the respective K_i values were calculated (see Ref 3). Mean values of n independent experiments and SEM are given
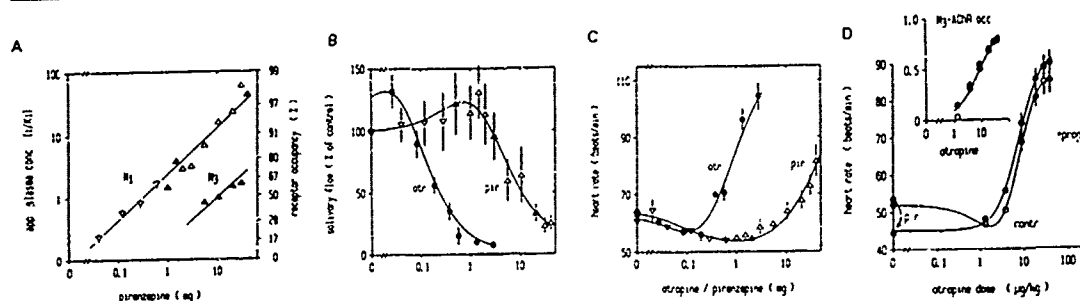
Fig 1 Comparison of mAChR subtype occupancy and dose–response curves of atropine (atr) or pirenzepine (pir) in healthy volunteers. In each session, volunteers (n = 9 to 12) received 6 to 9 consecutive i v bolus injections of drug at 20 to 30 minute intervals to cover cumulated doses of 0 02 to 40 mg for PZ or 0 4 to 40 µg/kg for atropine. Details of panels A–C have been published in Ref 5 and of D in Ref. 4. A: Radioreceptor assay of plasma samples after pirenzepine The respective mAChR subtype occupancy is depicted on the right ordinate. Apparent plasma concentrations (left ordinate) are given in pharmacological units, i e multifolds of the inhibitory dissociation constant (I/K,). (A 50% receptor occupancy thus coincides with a plasma concentration of 1 x I/K,) B and C. Dose-response of atropine or pirenzepine on salivary flow (B) and heart rate (C) under control conditions without β-blockade D Dose-response of atropine after β-blockade (240 mg of oral propranolol) without (open symbols) or with (closed symbols) pretreatment with an M, receptor blocking dose of pirenzepine (1.1 mg i.v) The inset shows the respective M$_3$ receptor occupancy obtained from the RRA of plasma samples. It can be inferred from the data in the inset that the pretreatment with PZ had no effect on M$_3$ receptor occupancy by atropine. (Figures reproduced with kind permission by Springer-Verlag)

However, the effects of PZ were not abolished by β-blockade as would be expected for an effect produced through a β-adrenergic pathway[4,5]. CNS effects are highly unlikely to be due to the high hydrophilicity and low permeation of PZ into the CNS. An intrinsic activity of PZ at postsynaptic receptors can be ruled out from studies in heart-transplanted patients[5]. These patients keep their sinus node fully innervated and receive an additional sinus node with the donated heart. Both atropine and pirenzepine showed no significant effect on the non-innervated sinus node in the donated heart. The recipients' sinus nodes, however, retained responsiveness to the antagonists comparable to healthy volunteers. These data and considerations led us to suggest that the cholino-mimetic effects of a peripheral M₁ receptor blockade are brought about by the blockade of inhibitory M₁ autoreceptors[4,5]. According to this hypothesis, block-

ade of these receptors by low dose PZ increases the postsynaptic cholinergic stimulus due to the interruption of a negative autofeedback mechanism.

Functional distinction between cardiac and glandular M-AChR in humans using AF-DX 116

AF-DX 116 has an affinity profile clearly distinct from PZ[2]. We administered 240 mg orally to healthy volunteers and monitored heart rate, salivary flow and accommodation capacity of the eye every 20 to 30 minutes. Plasma samples drawn in parallel were assayed in the above described RRAs. Atropine served as a control. Whereas atropine inhibited salivary flow almost completely and resulted in blurred vision, AF-DX 116 did not affect the accommodation capacity of the eye and induced a slight increase in salivary flow (Fig. 2). Atropine and AF-DX 116 increased the heart rate to a similar extent. Analysis of the RRA data is in

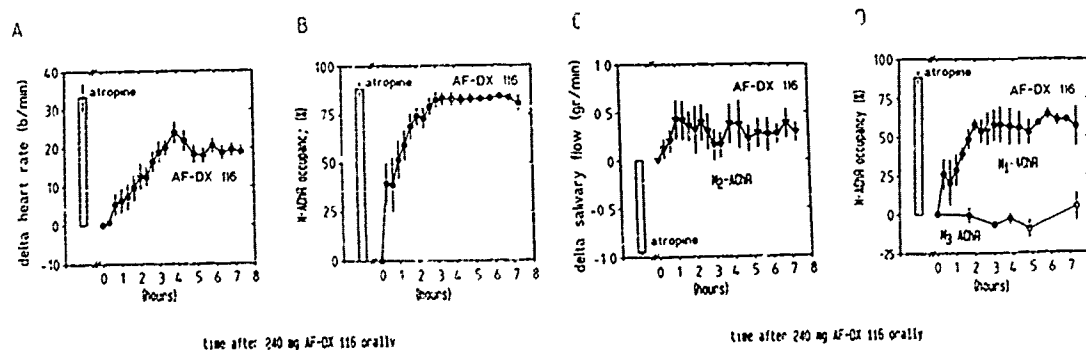

Fig. 2 Time–response curve after oral administration of 240 mg of AF-DX 116 to healthy volunteers (n = 7) 40 µg/kg of atropine served as a control. Heart rate (A) and salivary flow (C) were monitored and plasma samples were drawn in parallel From the respective RRA of plasma samples (see text for details), the mAChR subtype occupancies in vivo were estimated Original data published in Ref. 6.

good agreement with this effect pattern in the volunteers: M_2 receptors showed the highest occupancy by antagonist present in the plasma samples. The long-lasting steady-state tachycardia is matched by a sustained receptor occupancy in the *in vitro* assay. M_3 receptor occupancy was not detectable in agreement with no inhibitory effect of AF-DX 116 on salivary flow. The intermediate occupancy of the M_1 subtype seems to be matched by the slight increase of salivary flow. This interpretation though still speculative seems to fit with the above discussion of inhibitory M_1 autoreceptors: the effect of their blockade does not become apparent in the heart due to the overwhelming blockade of the postsynaptic M_2 subtype by AF-DX 116. However, in the salivary gland, the cholinergic stimulus becomes apparent as hypersalivation due to a missing postsynaptic blockade (for further data see Ref. 6).

Studies using i.v. administration of AF-DX 116 confirmed these results with respect to its pharmacodynamics. The pharmacokinetic behavior of the drug, however, was strikingly different: an elimination half-life from plasma of 2.5 to 3 hours was derived from the RRA of plasma concentrations and HPLC detection of AF-DX 116 i.v. (HPLC analysis was kindly performed by the laboratory of Prof. E. Mutschler, Frankfurt). The HPLC detection of AF-DX 116 in the plasma samples after oral drug administration also yielded sustained high plasma concentrations comparable to the results from the RRA. These data rule out a significant role of an active metabolite and suggest that perturbance of drug release and/or absorption of the oral preparation is responsible for this unique behavior. Further studies are being carried out at present.

With respect to clinical applications, the pharmacokinetic and pharmacodynamic profile of oral AF-DX 116 makes it a good tool for the treatment of bradycardia avoiding side effects associated with rather non-selective antagonists of the atropine type.

Sympathetic/parasympathetic interaction and M_1 receptors

Due to the close interconnections between sympathetic and vagal pathways we sought to understand regulatory functions of mAChRs with different mechanisms of sympathetic stimulation and compared isoprenaline- and exercise-induced tachycardia during mAChR subtype-selective blockade. It was demonstrated earlier that vagal effects in mammalian atria were more pronounced upon pretreatment of the preparation with isoprenaline[10]. This observation can be mimicked in humans as shown in Fig. 3A. The deceleratory dose–response curve of PZ maintains virtually the same ED50 comparing not stimulated (Fig. 1C) and isoprenaline-stimulated conditions (Fig. 3A). The amplitude of the effect, however is increased twofold. Tachycardia induced by exercise shows a different effect pattern with an M_1 receptor blocking dose of PZ. As shown in Fig. 3B (left side), the deceleratory effect of PZ disappears during exercise-induced tachycardia obviously due to the reduced vagal activity with increasing physical stress[11,12]. During the recovery phase (Fig. 3B, right side) this effect of the M_1-selective blockade becomes apparent again with
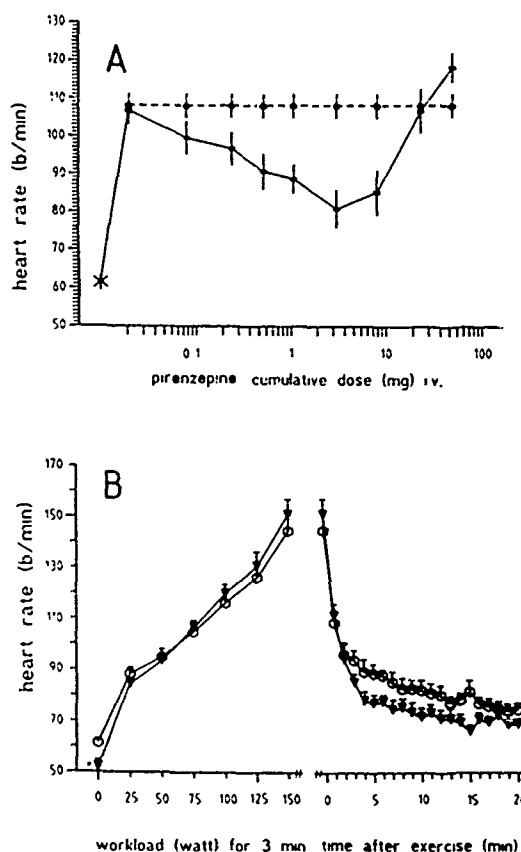
Fig 3 Interaction between M_1 receptor blockade and sympathetic stimulation in healthy volunteers Isoprenaline-(A) and exercise-induced tachycardia (B) A Dose–response of pirenzepine on the isoprenaline-induced tachycardia (n = 9) The individual isoprenaline dose necessary to increase the heart rate to about 110 beats/min was titrated and kept constant for 3 5 hours (22 1 ng x min^{-1} x kg^{-1}) Carrier only (dotted line) or cumulative doses of PZ (0 08 to 49 mg i v, heavy line) were administered at 20 to 30 min intervals B Effect of low dose PZ on exercise induced tachycardia Volunteers (n = 6) received either placebo (o) or PZ (triangle) and underwent an increasing work load (25 watts every 3 min) The heart rate was monitored during this exercise and during a 20 minute recovery phase Before exercise (0 time point) and 4 to 17 min after exercise, a significantly lower heart rate was observed in the PZ group (p < 0 05)

the reappearance of vagal activity These two independent results also support the notion of a peripheral M_1 receptor functioning as an inhibitory autoreceptor in humans. Furthermore, the different regulation of vagal activity during exercise tachycardia in comparison with isoprenaline-induced tachycardia can be unmasked by the PZ induced M_1 blockade at 110 beats/min PZ decreases the isoprenaline-stimulated heart rate significantly by 30 beats/min, whereas at the same heart rate due to physical exercise PZ remains without effect (Fig 3 panel A versus B).

Perspectives

The data from these studies in healthy human volunteers led us to design a combination of exercise and isoprenaline tests in combination with low dose PZ for the detection of latent sympathetic/vagal mismatches in young patients with syncopic attacks. In addition, we employ this combination of selective cholinergic and sympathetic challenge in the diagnosis of latent intracardiac conductivity problems. Furthermore, we believe that AF-DX 116 should be a good alternate tool for the long-term treatment of vagally induced bradycardias due to its favorable selectivity profile *in vivo*.

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Abstracts of Poster Presentations

1 Pharmacokinetics and effects of single oral doses of the novel selective antibradycardic agent AF-DX 116 BS in normal subjects

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Chronic treatment of bradycardia using anticholinergics such as atropine is limited by their side effects; the current therapeutic alternative is pacemaker insertion. A new promising, selective antibradycardic agent is AF-DX 116 BS, a cardiospecific M2 cholinoceptor antagonist.

In a double blind randomized Phase I study, 4 groups of healthy male volunteers were given single oral doses of placebo (P), 120 mg (D1), 240 mg (D2) or 480 mg (D2) of AF-DX 116 BS. Peak plasma concentrations analysed by HPLC were 84 (P1), 370 (D2) and 920 (D3) ng/ml; AUC values were 750 (D1), 2710 (D2) and 7760 (D3) ng.h/ml. t(max) was 2.3-2.6 h and plasma levels subsequently decreased only slowly (mean residence time 12.5-15.3 h).

Mean baseline heart rate (HR; 1/min) was 64± 7 [SD] (P), 61±8 (D1), 64±6 (D2) and 64±7 (D3) [n.s.]. Maximum increases in HR were ¯3±5 (P), 4±5 (D1; p<0.1 vs P), 15±7 (D2; p<0.01 vs P), and 21 ± 9 (D3; p < 0.001). Median duration of action was 3.2 h (D1), 14 h (D2) and 22.7 h (D3). Salivary flow, near-point vision and pupil diameter were unaffected.

In conclusion, AF-DX 116 BS is efficacious and well tolerated after single oral doses, shows a prolonged duration of action and is potentially suitable for chronic administration.

2 Clinical pharmacology of AFDX 116 BS, a novel selective antibradycardic agent after repeated dosing in normal subjects

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Conventional treatment of bradycardic arrhythmias comprises pacemaker insertion or therapy with anticholinergic or beta-mimetic drugs such as atropine and orciprenaline. The use of these agents is however limited by their side effects. A promising new entity for this indication is AFDX 116 BS, an M2-cholinoceptor antagonist which has demonstrated high cardio-selectivity in in vitro receptor binding studies and after single doses in healthy volunteers.

In this double blind randomized study 3 groups of 12 healthy male volunteers received oral doses of placebo (P), 120 mg (D1) and 240 mg (D2) AFDX '16 BS twice daily over 5 days.

Baseline heart rates (HR; 1/min) were (mean ± SD): 63 ± 11 (P), 66 ± 9 (D1) and 66 ± 10 (D2) (n.s.). Average increases in HR on day 5 over the 12h period following the last dose were 0 + 5.3 (P), 6.3 ± 6.8 (D1; p<0.1) and 11.8 ± 5.8 (D2; p<0.001).

The medication was well tolerated, with no significant changes in salivary flow, near-point vision and surface ECG.

Pharmacokinetic steady state was effectively reached after 2 days, with minimum plasma levels of 46 ± 16 ng/ml (D1) and 117 ± 43 ng/ml (D2). C_max was 247 ± 224 ng/ml (D1) and 590 ± 232 ng/ml (D2), terminal half life 15 ± 4h (D1) and 12 ± 1.3h (D2). The accumulation factor was low (1.6), and kinetics were linear over the dose range studied.

In conclusion, the efficacy, selectivity and pharmacokinetics of AFDX 116 BS after repeated dosing are highly favorable and justify the initiation of trials in patients with bradycardia.

3 Muscarinic agonists for senile dementia. past experience and future trends

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A role for muscarinic receptor agonists in the symptomatic treatment of Alzheimer's Disease is suggested by the pathological findings in postmortem brain tissue of pre-synaptic cholinergic neuronal loss combined with preservation of post-synaptic M1 receptor number

Classical muscarinic agonists have mixed activity at M1 and M2 receptors Examples of such agents include pilocarpine, oxotremorine, arecoline and bethanechol (the latter given intracerebroventricularly), which have produced generally equivocal or negative results in small clinical trials

RS 86, also a mixed M1/M2 receptor agonist, produced a positive clinical response in 15 per cent of patients in 3 out of 5 clinical studies Responders could not be characterised retrospectively based on clinical observations

However, the action of these agents at M2 receptors leads to 1) peripheral cholinergic effects, which may be dose-limiting and possibly 2) a decrease in neuronal release of acetylcholine, an effect which may be partially offset by M2 receptor loss in brain of Alzheimer's Disease patients.

More potent and better tolerated muscarinic agonists are needed for clinical studies Theoretical approaches include the design of highly lipid soluble agents to allow rapid brain penetration and hence a higher central to peripheral effect ratio, a search for specific M1 or M1-subtype selective receptor agonists, using cloned specific receptor subtypes, and the development of pre-synaptic muscarinic antagonists to facilitate acetylcholine release and hence stimulate both muscarinic and nicotinic receptors

4 Muscarinic receptor alterations in human cognitive disorders

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The literature contains many discrepancies concerning the status of muscarinic receptors in human neurodegenerative diseases. In the present study, we characterized the binding of various muscarinic ligands to homogenates of both cortical and subcortical regions of Alzheimer's (AD). Parkinson's (PD), and Parkinson's with AD-type dementia (PD/AD) brains. Our results suggest that the total density of muscarinic receptor sites, assessed using ^{3}H-QNB as ligand, is not altered by disease, except in the caudate-putamen, where the Bmax is increased This increase in the density of muscarinic sites may be due to an up-regulation of the M1-subtype, since we found the Bmax for ^{3}H-pirenzepine binding was similarly enhanced. Conversely, the density of muscarinic-M2 sites was reduced in all areas in the PD/AD and AD brains, and in the striatum and hippocampus of PD brains. This decrease in ^{3}H-AF-DX 116 or 3H-ACh binding in cortical tissue ranged from 38-61% and was significantly correlated with the reduction in choline acetyltransferase (ChAT) activity in these same brain regions, at least in AD. Therefore, it appears that muscarinic-M2 receptor sites in cortical regions and in hippocampus, like ChAT, may be localized to presynaptic cholinergic terminals. In summary, the density of muscarinic receptor sites is differentially altered in various regions of the human brain and the degree of deficit appears to depend on the type of neurodegenerative disease involved, as well as its severity.

5 i.c.v. AFDX-116 induces analgesia only when administered at very low doses

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Antinociception can be induced in animals by stimulating central muscarinic receptors. This effect can be obtained either by direct muscarinic agonists or by indirect cholinomimetics such as cholinesterase inhibitors and specific antagonists of muscarinic presynaptic autoreceptors, such as atropine, given at very low doses(1) and local anaesthetics(2). Since many authors have reported that in the CNS cholinerg c inhibitory autoreceptors are of M_2 subtype, we decided worthwile to test the analgesic effect of AFDX-116, a selective M_2 antagonist. AFDX-116 (6.3 ng/mouse i.c v.) was able to induce a statistically significant antinociception in mice tested on hot-plate and writhing tests. AFDX-116 doses ten times respectively higher and lower than the analgesic one were ineffective. AFDX-116 antinociception was antagonized by atropine (5 mg/kg i.p.) and by HC-3 (1 µg/mouse i.c.v.). Naloxone (1 mg/kg i.p.) did not modify AFDX-antinociception. Analgesia induces by AFDX-116 was obtained without any adverse symptomatology as resulted also in experiments using the rota-rod test. AFDX-116 therefore was able, like very low doses of atropine (1) and local anaesthetics (2), to induce antinociception through cholinergic activation. Experiments are now in progress to elucidate why the higher doses of AFDX-116 were not effective.

Bartolini et al. X IUPHAR, Sydney, P36 P816 (1987)
Bartolini et al. Br. J. Pharmac. 92, 711 (1987)

6 Analgesia induced by the M_2 antagonist methoctramine administered i.c.v.

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Antinociception can be induced in animals by stimulating central muscarinic receptors. This effect can be obtained either by direct muscarinic agonists or by indirect cholinomimetics such as cholinesterase inhibitors and specific antagonists of muscarinic presynaptic autoreceptors, such as atropine given at very low doses (1) and local anaesthetics (2). Since many authors have been reported that in the CNS cholinergic inhib tory autoreceptors are of M_2 subtype we thuoght it worthwile to test the analgesic effect of methoctramine, a selective M_2 antagonist. Methoctramine (5-100 ng/mouse i.c.v.) was able to induce a statistically significant antinociception tested in hot-plate and writhing tests. Methoctramine antinociception (10 ng/mouse i.c.v.) was completely antagonized by atropine (5 mg/kg i.p.) and by HC-3 (1 µg/mouse i.c.v.) but not by naloxone (1 mg/kg i.p.) and α-methyl-p-tyrosine (100 mg/kg i.p.).Moreover the i.c.v. injection of antinociceptive doses of methoctramine did not impair performance on the rota-rod test. On the basis of tnese results and of the exsisting literature we hypothesized that methoctramine like atropine at very low doses and local anaesthetics, induces antinociception by a facilitation of cholinergic transmission.

1) Bartolini et al. X IUPHAR Sydney, P36 P816 (1987)
2) Bartolini et al. Br. J. Pharmac. 92, 711 (1987)

7 Muscarinic M_1 contrary to M_2 antagonists do not induce analgesia

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Atropine (1-100 µg/kg s.c.: 1-10 ng/mouse i.c.v.)(1),AFDX 116 (6.3 ng/mouse i.c.v.)(2) and methoctramine (10-50 ng/mouse i.c.v.) (3) are able to induce antinociception when administered at very low doses. Since their analgesic effect is antagonized by atropine (5 mg/kg i.p.) and by HC-3 (1 µg/mouse i.c v.) it appears possible that such an effect might be due to an indirect activation of the central cholinergic system. Moreover since oxotremorine analgesia is antagonized by atropine but not by HC-3,a direct mechanism of action like that of oxotremorine may be ruled out for the three antimuscarinic drugs. On the basis of these in vivo experiments together with results obtained in vitro (1) we concluded that AFDX-116, methoctramine and atropine induce antinociception by blocking selectively the inhibitory presynaptic autoreceptors. Since M_1 subtype of muscarinic receptors were described also at the presynaptic level (4), pirenzepine and dicyclomine were tested for analgesia too. Although a large range of doses was tested neither pirenzepine nor dicyclomine administered either i.c.v. or i.p. were able to induce analgesia in mice tested on hot-plate and writhing. These results are in agreement with most literature reports showing that muscarinic autoreceptors are of M_2 subtype.

1) Bartolini et al. (1987) X IUPHAR, Sydney P36 P816
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3) Gualtieri et al. this meeting
4) Suzuki et al. Neurosci. Lett. (1988) 84, 209.

8 In-vivo selectivity of muscarinic agonists with a 1,3-oxathiolane nucleus

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The cardiovascular effects of the muscarinic agonist (±) cis-2-methyl-5-(dimethylamino)methyl-1,3-oxathiolane methiodide (±I) and its two enantiomers have been investigated in the pithed rat. Muscarine served as a reference drug All compounds produced brief decreases in heart rate and mean arterial pressure (mediated by M_2 receptors) followed by a more sustained tachycardia and pressor response (mediated by ganglionic M_1 receptors). The two enantiomers displayed pronounced enantioselectivity, the (+) enantiomer being 18-42-fold more potent (Tab). Muscarine showed similar potencies in eliciting both the inhibitory and excitatory actions, and thus did not discriminate between M_1 and M_2 receptors. In contrast, racemic oxathiolane (±I) and both enantiomers displayed a sligth (3-7-fold) selectivity for the excitatory M_1 as compared to the inhibitory M_2 responses. It thus appears that muscarinic agonists carrying a 1,3-oxathiolane nucleus may be useful tools in the development of more selective M_1 receptor agonists.

| | ED_{50}(nmol/Kg) Pithed rat | | | |
| | Decrease in | | Increase in | |
	heart rate	MAP[a]	heart rate	MAP[a]
muscarine	16.7	19.1	11.9	20.5
(±)I	8.9	8.6	2.6	2.6
(-)I	.45	.3	....	23...
(+)I	4.6	4.3	0.6	0.6

[a]MAP = mean arterial pressure

9   CI-969: An orally active muscarinic receptor agonist related to arecoline

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Muscarinic agonists have been suggested to be useful in the treatment of Alzheimer's disease. In this report we present the characterization of the muscarinic receptor agonist, 1-'1,2,5,6-tetrahydro-1-methyl-3-pyridinyl) - ethanone, O-acetyloxime, HCl (CI-969). In vitro, CI-969 binds with high affinity to rat brain muscarinic receptors with no significant binding at a number of other receptors. Stimulation of scopolamine-sensitive phosphatidylinositol turnover was observed in rat cortical slices and SK-N-SH cells, while presynaptically CI-969 decreased both the release of [^{3}H]-ACh from rat cortical slices and high affinity choline uptake. In vivo, CI-969 produced dose-dependent cholinergic effects in models of central (body temperature, EEG, and local cerebral blood flow) and peripheral (GI motility, heart rate) cholinergic activity with rodents showing activity in the range of 1-3.2mg/kg and non-human primates at 0.01mg/kg. In addition, analgesic efficacy was also observed. Thus, CI-969 is a novel muscarinic receptor agonist showing central activity upon oral administration.

10   Transfer of ACh-like behaviours from CER to 3 paradigms

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Previous studies from this laboratory have demonstrated that the presentation of a conditioned stimulus (CS) to rats elicits conditioned emotional response (CER). This acquisition and extinction of behavior is correlated with a hypercholinergic-like state which results in compensatory muscarinic binding site plasticity in the cerebral cortex. We tested the hypothesis that drug-naive rats exposed to CER would behave in other well-characterized behavioral paradigms as though they had received cholinergic agonists. Groups of animals were trained on a CER schedule, such that presentation of CS on testday suppressed food-reinforced responding and produced collateral behaviors reminiscent of emotion/anxiety. Directly after the CER session, individual animals were tested for passive avoidance, active avoidance, or activity (Digiscan apparatus). Compared to controls (no CS presentation), CER rats exhibited (statistically significant, p<0.05): a 24 percent decrease in locomotor activity, a 75 percent increase in stereotypy, a 50 increase in antithigmotactic behavior, a 15 percent increase in trials to criterion for active avoidance, and a 27 percent decrease in trials to criterion for passive avoidance. Numerous other activity measures did not distinguish the groups. A small number of animals which failed to exhibit CER did not demonstrate the changes in activity and learning/memory that were otherwise observed in CER animals. Drug-naive CER rats show decreased locomotor activity, facilitated passive and retarded active avoidance, just as though the animals had received muscarinic agonists or acetylcholinesterase inhibitors. (supported by DAMD-17-88-C8026)

11   Relative aversive potency of muscarinic agonists

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Cholinomimetics with low aversive activity (e.g. nausea/emesis) should be useful for dementia therapy. Conditioned taste aversion (CTA) was used to estimate the potential of drugs to induce such aversive effects. The potencies of full and partial agonists and anticholinesterases were assessed for induction of CTA in rats and for other CNS effects in mice e.g. inhibition of exploratory locomotion; induction of hypothermia and tremor (see table below).

		ED$_{50}$ mg/kg sc		
Drug	CTA	locomotion	temperature	tremor
BRL 47042*	0.0005	0.0002	0.0006	0.002
eserine	0.29	0.05	0.1	0.1
oxotremorine	0.3	0.02	0.04	0.2
BM5	0.59	0.07	0.7	10 I
THA	1.8	1.58	6.0	6.0
pilocarpine	5.8	1.57	7.0	10 I

I=inactive, *BRL 47042 is exo-3-(3-methyl-1,2,4-oxa-diazol-5yl)-azabicyclo[2.2.1]heptane

All cholinomimetics tested caused CTA at doses higher than those that inhibited exploratory locomotion, an effect taken to reflect the cognitive potential of the drugs. The partial agonists BM5 and pilocarpine did not induce tremor, unlike the full agonists, BRL 47042 and oxotremorine which did. Low tremorogenic activity of these partial agonists may not be a real advantage. It is offset by their potency in CTA which suggests that aversive side effects will be the more limiting factor.

12   Carbachol induces an inward Na$^+$ current in ventricular myocytes by activating M$_2$ muscarinic receptors

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Carbachol induces inward current carried by Na through TTX-resistant channels in ventricular and atrial cell membranes. This action of carbachol, which is shared by acetylcholine but not by oxotremorine, is prevented by the non-selective muscarinic receptor (mAChR) antagonist, atropine. Voltage clamp experiments were done on isolated guinea pig ventricular myocytes in Cs$^+$ (20mM) - containing Tyrode solution to ascertain the pharmacological properties of the mAChR involved in the action of carbachol. Carbachol (300μM) induced a steady inward current of ~25 pA when the membrane was clamped at the resting potential (-75mV). Atropine, AFDX-116 (M$_2$-selective) and pirenzepine (M$_1$-selective) suppressed the carbachol-induced inward current in a concentration-dependent manner. The antagonist concentrations for 50% inhibition (IC$_{50}$) of the carbachol effect were 20nM, 200nM and 1500nM for atropine, AFDX-116 and pirenzepine, respectively. The results are consistent with the hypothesis that the inward current by carbachol is initiated at M$_2$ rather than M$_1$ receptors. The M$_1$-selective agonist, McN-A-343 (300μM), did not induce an inward current and could antagonize that evoked by an equimolar concentration of carbachol. Summarily, the results indicate that the inward Na current induced by carbachol, like the outward K current usually evoked by this agonist, arises from activation of M$_2$ receptors. The increased entry of Na$^+$ caused by carbachol, which may stimulate Na-Ca exchange, is responsible for the atropine-sensitive positive inotropic effect seen in ventricular muscle from reserpine-treated guinea pigs.

13 Effect of muscarine on membrane properties of locus coeruleus neurons

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The effects of muscarinic agonists were measured by intracellular recordings of membrane potential and membrane current (single electrode voltage clamp) of single locus coeruleus (LC) neurons in slices of rat pons. Muscarinic agonists produced three distinct effects. These were: 1) A direct excitation via activation of M2 (cardiac) receptors (Christie, M.J. and North R.A. (1988) Trends Pharmacol. Sci Suppl. Subtypes of Muscarinic Receptors III, P89); 2) inhibition of a non-inactivating calcium current; 3) reduction in the potassium current caused by flow by agonists at μ-opioid or α2-receptors. In normal superfusion medium the direct excitation was not associated with a change in membrane conductance or involvement of potassium conductance. Under conditions in which most types of currents were blocked, muscarine produced an inward current with increased membrane conductance which reversed polarity at -4 ± 3 mV (n=4), suggesting the involvement of increased non-selective cation conductance. Pre-treatment of animals with pertussis toxin did not abolish the inward current (~60 $\pm$ 6 pA at -60 mV, n=3). Superfusion of slices with phorbol 12,13-diacetate did not mimic or affect responses of the first or third type. It is intriguing that the muscarinic excitation of LC neurons is mediated by M2 receptors; although these cells have other receptors (μ and α2) that couple to potassium channels, the M2 receptors fail to do so and instead excite the cells through an unknown second messenger. This second messenger might also bring about the impairment of coupling between μ and α2 receptors and the potassium channels and perhaps also the reduction in calcium current.

14 M₁ and M₃ muscarinic receptors mediate two types of membrane electrical responses in *Xenopus* oocytes

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Native Xenopus oocytes exhibit two types of responses to acetylcholine (ACh). The common response in oocytes of a majority of donors consist of a rapid, transient (D1) and a prolonged, slow depolarizing chloride current (D2). Oocytes of rare variant donors exhibit a similar two-component depolarizing chloride response. Despite this similarity, these two types of responses exhibit profound differences of amplitude, kinetics, sensitivity to activation of protein kinase C and to collagenase treatment and, strikingly, differences of sensitivity of the two hemispheres of the oocyte to local application of agonist (see Table).

	Common	Variant
Responses at the two hemispheres (animal/vegetal)	0.7	5
Latency (sec)	0.9	5.4
Amplitude (nA)	<300	>1000
45-Ca efflux (% of total)	2.6	30
Effect of phorbol esters	enhance	inhibit
Effect of collagenase	inhibit	enhance

Preliminary evidence indicates that the common response was mediated by M3 and the variant response by M1 receptors. As variant responses closely resemble acquired responses to ACh in oocytes that express receptors after injection of foreign mRNA and use the inositol trisphosphate-calcium signal transduction pathway, we suggest that they are an intrinsic model for this class of responses in native Xenopus oocytes.

15 Muscarinic receptors mediating phosphatidylinositol turnover in cultured smooth muscle cells of the rabbit aorta

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Acetylcholine (ACh) induced a concentration-dependent accumulation of inositol phosphate (IP₁) formation in cultured smooth muscle cells of the rabbit aorta. The effects of three muscarinic antagonists, namely atropine, scopolamine, pirenzepine and a GTP analogue (GppNHp) on the ACh-induced accumulation of IP₁ were investigated. The four compounds inhibited concentration-dependently the ACh-induced accumulation of IP₁. The IC_{50} values for atropine, scopolamine, pirenzepine and GppNHp were 22 nM, 440 μM, 3.7 μM and 35 nM respectively. The data seem to indicate that the muscarinic receptors mediating the breakdown of phosphatidylinositol in the smooth muscle (i) have extremely low affinity for scopolamine, (ii) are unlikely to be the M₂ types of receptors and (iii) are GppNHp sensitive. The data also support our recent findings that the muscarinic receptors in the smooth muscle of the rabbit aorta consist of a high-(GppNHp) sensitive and a low-affinity binding site (1). Our latest study also show that the binding of ^{3}H-ACh to the smooth muscle muscarinic receptors is displacable by nanomolar range of atropine but only by micromolar range of scopolamine.

(1) M.K. Sim & S. Manjeet. Eur. J. Pharmacol (in Press).

16 Are there multiple types of muscarinic receptors linked to inositol-monophosphate formation in rat hippocampus?

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Muscarinic antagonists, non-selective or putatively selective for M1, M2 or M3, receptors, were tested against carbachol-induced inositol-monophosphate (IP1) formation in slices of rat hippocampus. The classical antagonists, atropine and N-methylscopolamine, had Hill coefficients > 0.9, consistent with interaction at one site. The M1 selective analogs pirenzepine and telenzepine differed in their Hill coefficients (Nh), the former had a value of 0.' while the latter was 1.03. The Nh of the M1 selective analog of pirenzepine, AF-DX 116,, was also close to unity. Hexahydrosiladifenidol, a M3 selective antagonist, inhibited IP1 formation with Nh = 0.7 while for its carbon analog, trihexyphenidol, Nh = 0 8. Dicyclomine, which also displayed high affinity at the M3 site, had a low Hill coefficient (Nh = 0.5) Himbacine, selective for M2 receptors but with similar low affinities at M1 and M3 sites, also blocked IP1 formation in rat hippocampus slices with a low Nh (0.7). Differences were also found between agonists. Carbachol, oxotremorine-M and arecaidine propargyl ester were full agonists in stimulating IP' formation but, whereas the former two gave a monophasic concentration-response curve, the latter gave a biphasic response. Thus, stimulation of IP1 formation in rat hippocampus appears to be linked to more than one type of muscarinic receptor. The affinity values obtained with the above antagonists suggest these to be a mixture of M1 and M3 receptors.

17 Cholinergic stimulation of phosphoinositide breakdown and detrusor smooth muscle contraction in absence of extracellular calcium

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Previous studies showed that muscarinic receptor-mediated phosphoinositide breakdown (PI) may serve as the transducing mechanism for cholinergic contraction of the urinary bladder (Noronha-Blob et al., JPET, 1989). Furthermore, we showed that it is the anticholinergic activity of agents clinically used to treat urinary incontinence that is important in controlling bladder contractility and voiding, although these drugs may also possess other ancillary activities (Noronha-Blob et al, 1988).

In the present study, guinea pig detrusor smooth muscle contraction was induced by carbachol (CBC) in the absence of extracellular calcium ($[Ca^{2+}]_o$) with a lower efficacy (~60%) but with the same potency (EC_{50} ~2 μM) as that observed in presence of $[Ca^{2+}]_o$. In contrast, KCl-evoked contractions were abolished in absence of $[Ca^{2+}]_o$, but returned when Ca^{2+} was restored. Clinically useful drugs (oxybutynin, imipramine, dicyclomine, propantheline and terodiline) produced rightward parallel shifts of the CBC dose response curve yielding K_b values that were similar both in presence and absence of $[Ca^{2+}]_o$. Phorbol 12,13-dibutyrate (PDBu), an activator of protein kinase C, but not 4-α-PDBu, caused detrusor contractions in presence of $[Ca^{2+}]_o$ only. CBC also stimulated PI turnover both in presence and absence of $[Ca^{2+}]_o$. Together, these findings suggest that (1) detrusor contractions may occur in absence of $[Ca^{2+}]_o$ presumably by inositol phosphate-induced intracellular Ca^{2+} mobilization; (2) since drugs used for urinary incontinence block both PI turnover and smooth muscle contraction in absence of $[Ca^{2+}]_o$, their pharmacological mechanism of action may be mediated through the coupling of muscarinic receptors to the PI transduction system.

18 Muscarinic receptor mediated hyperpolarization of somata membrane in toad dorsal root ganglion neurons and its underlying ionic basis

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It has been reported that there exist ACh receptors, both nicotinic and muscarinic receptors, in somata membrane of dorsal root ganglion (DRG) neurons (Morita & Katayama., Brain Res.,44, 273, 1984). In our experiments, intracellular recordings were performed from isolated superfusion preparation of toad DRG neurons. In total of 70 neurons, 65 were of type A cell and remaining 5 were of type C cell. During the application of 2×10^{-4}M ACh into the recording chamber by dropping, a hyperpolarization or a biphasic response (an initial hyperpolarization followed by a more pronounced depolarization) results, which was blocked by pre-perfusion of 1.3×10^{-5}M atropine (n=25). Besides, the depolarization could be antagonized by pre-perfusion of 1.4×10^{-5}M tubocurarine (n=18). The hyperpolarization may also be induced by application of muscarine, the response was blocked by gallamine but not by pirenzepine. This indicates that the soma of toad DRG neuron is endowed with M_2 receptor. In further study of ionic basis in muscarinic hyperpolarization, an increase in membrane potassium conductance and a value of average -96mV in reversal potential were observed during the change in membrane potential, which can be blocked or partial blocked by perfusion of TEA and $BaCl_2$ or by removal of extracellular calcium. These results suggest that the activation of calcium-sensitive potassium conductance ($g_{k,ca}$) might be involved in muscarinic hyperpolarization.

19 Preferential activation of calcium/calmodulin kinase rather than protein kinase-C (PK-C) by M_3-receptors in human colonic epithelial carcinoma cells (HT 29)

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Human colonic mucosa contains mainly M3-receptors. We investigated details of their coupling to the phosphatidyl-inositol (PI) second messenger system in human colonic HT-29 cells which contain 4000 M3-receptors/cell. The PI-system generates the 2 second messengers, inositol trisphosphate which elevates intracellular calcium and diacylglycerol (DAG). These second messengers in turn activate either Ca/calmodulin kinase or PK-C which may lead to different responses.

Muscarinic stimulation with carbachol (0.1 mM) increased formation of (.H)IP3 and caused a brief early and a delayed increase in DAG (determined radioenzymatically). In unstimulated cells PK-C was primarily (90%) in the soluble compartment and there was only a small translocation to the membrane compartment upon muscarinic stimulation (10%). To obtain direct evidence for activation of either Ca/calmodulin kinase or PK-C, labeling of phosphoproteins with (..P)i upon stimulation with carbachol or TPA was determined. Phosphoproteins were resolved by two dimensional polyacrylamide gel electrophoresis and visualized by autoradiography. At 15 min TPA reproducibly altered the phosphorylation of at least 9 proteins. Carbachol altered the phosphorylation of 5 proteins of which only one coincided with those affected by TPA.

We conclude that cholinergic stimuli primarily activate the Ca/calmodulin response branch while PK-C appears to play only a minor role. The mechanisms of this differential regulation are presently under investigation.

20 Protein kinase-C is not involved in muscarinic phosphoinositide feedback regulation in human intestinal cells

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HT-29 colon carcinoma cells contain M3 muscarinic receptors coupled to phosphoinositide(PI)-metabolism. Pretreatment of these cells with carbachol (0.1mM, 4 h) inhibits the subsequent ability of carbachol to increase [^{3}H]-inositolphosphate(IP) accumulation, which indicates desensitization. Although activation of endogenous proteinkinase-C seems to be involved in desensitization of PI-metabolism, the mechanism in intestinal cells is unknown. We investigated agonist and proteinkinase-C (PK-C) mediated desensitization by determination of muscarinic binding sites, NaF-stimulated IP-release and direct measurements of phospholipase C (PL-C) activity.

Results: Agonist induced desensitization is paralleled by a complete loss of muscarinic binding sites. In contrast, proteinkinase-C mediated desensitisation by incubations with phorbolesters (PMA 0.1 mM, 95% inhibition after 4 hours), is accompanied by an only 40% reduction of[^{3}H]NMS binding, suggesting an additional mechanism of negative feedback control. Membrane-associated PK-C activity increased only 10-20% after carbachol treatment (15 min). Direct stimulation of IP-release by NaF (20mM) was not affected after pretreatment with phorbolesters or carbachol, demonstrating an intact function of G-protein and PL-C at the effector side. Determination of PL-C activity using [^{3}H]P^7 as substrate, indicated no major effects after carbachol or PMA pretreatment.

Conclusion: Our results indicate that agonist-induced desensitization of PI-turnover mainly occurs at the receptor level with a rapid loss of muscarinic receptors. Activation of endogenous PK-C contributes only to a minor extent to negative feed-back control mechanisms in human intestinal cells.

21 Differential activation of inhibitory and facilitatory muscarine receptors on the rat phrenic nerve under different stimulation conditions

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Transmitter release from the motor nerve is under the local control of nicotine and muscarine autoreceptors. According to the potencies of pirenzepine and dicyclomine both types of muscarine receptors might be regarded as M1-receptors. In the present experiments activation of muscarinic autoinhibition or autofacilitation was investigated under different stimulation conditions: continuous nerve stimulation (current strength 8 mA; 100 or 1500 pulses at 5 Hz), intermittent nerve stimulation (40 pulses at 50 Hz every 10 s; 680 or 1500 pulses), continuous field stimulation (current strength 80 mA; 100 or 1500 pulses at 5 Hz). Increase or decrease by scopolamine of evoked [³H]acetylcholine (ACh) release indicates activation of muscarinic autoinhibition and autofacilitation, respectively.
Scopolamine enhanced ACh release evoked by the short period (100 pulses) of continuous nerve or field stimulation. The antagonist reduced ACh release evoked by the long period (1500 pulses) of nerve stimulation. Scopolamine failed to affect ACh release evoked by the long period of both field and intermittent nerve stimulation. However, scopolamine enhanced ACh release during intermittent nerve stimulation when the number of pulses was reduced (680 pulses). The present experiments demonstrate muscarinic autoinhibition which is activated during short periods of continuous or intermittent nerve stimulation. Muscarinic autofacilitation occurs during a long period of continuous nerve stimulation, whereas intermittent nerve stimulation or continuous field stimulation prevents muscarinic autofacilitation from being activated. It is proposed that the facilitatory muscarine receptors of the motor nerve are located proximately to the inhibitory receptors. The latter receptors appear to be localized near the active release zones.

22 On the muscarine receptors of cholinergic nerves and sphincter muscle in the isolated guinea-pig iris

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Muscarine receptors (MR) on sympathetic nerves of the rabbit or guinea-pig iris belong to the M_2 type whereas the postjunctional MR in the rabbit iris sphincter are of the M_3 type (1). To obtain information on the type of MR mediating inhibition of the acetylcholine (ACH) release, we investigated the overflow of ^{14}C activity after loading the cholinergic nerves of the guinea-pig iris with ^{14}C-choline. The increase by MR antagonists of ^{14}C overflow evoked by field stimulation (2 min, 5.5 Hz, 1 ms) was taken as a parameter for blockade of prejunctional MR. At the same time the decrease by MR antagonists of iris sphincter contractions upon evoked ACH release were taken as a measure for blockade of postjunctional MR.
Atropine 0.1 µM doubled the evoked ^{14}C overflow, abolished the initial phasic, and reduced the secondary tonic contraction of the iris. Likewise pirenzepine 10 µM maximally enhanced the evoked ^{14}C overflow (n=5) and decreased the contraction responses indicating the lack

of an M_1 MR at both sites. In contrast, the M_2 selective hexahydrosiladifenidol failed to affect the ^{14}C overflow up to 10 µM (n=5) but decreased the postjunctional responses at > 1 µM indicating the lack of a prejunctional M_2 MR. Gallamine 100 µM (n=5) enhanced both the ^{14}C overflow and the contraction. Similarly the M_2 selective methoctramine > 1 µM (n=4) significantly enhanced the evoked overflow and increased the postjunctional response to field stimulation.
The data are compatible with the idea that ACH released from the guinea-pig iris causes autoinhibition via M_2 MR and contracts the iris sphincter probably via M_3 MR.

23 On the prejunctional muscarine receptors of cholinergic nerves in the perfused rat heart

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Prejunctional muscarine receptors (MR) on noradrenergic nerves and postjunctional MR in the rat heart belong to the M_2 or cardiac type (1). We studied the effects of several MR antagonists on the autoinhibition of acetylcholine (ACH) release in the perfused rat heart with both vagal nerves attached. The ACH stores were pulse-labelled with ^{14}C-choline, and the tetrodotoxin-sensitive ^{14}C-overflow into the perfusate upon 7 vagal stimulations (S, 10 min apart; 720 pulses, 10 Hz) was determined. Stimulation-evoked overflows in the absence or presence of the antagonists (14 or 4 min equilibration) are given as S4 or S5/S2 ratios (** P < 0.01). Concentrations of antagonists were chosen to yield about 10 times the pA_2 at the cardiac (AF-DX 116) or at the ileal MR type (hexahydrosiladifenidol, HHSiD).

	(µM)	S4/S2	S5/S2	n
Controls		0.90 ± 0.11	0.59 ± 0.11	6
Atropine	(0.001, 0.01)	1.31 ± 0.27	1.87 ± 0.35**	5
AF-DX 116	(0.1, 1)	1.90 ± 0.09**	1.63 ± 0.10**	5
HHSiD	(0.01, 0.1)	0.95 ± 0.17	0.75 ± 0.13	5

Atropine 0.01 µM increased the evoked ^{14}C-overflow about 3 fold compared to controls, indicating that under these stimulation conditions, the autoinhibition is activated by endogenous ACH. The cardio-selective antagonist AF-DX 116 was about 100 fold less potent than atropine. At concentrations selective for the ileal type of MR, HHSiD failed to affect the ACH release. The results are compatible with an M_2 or cardiac type of MR mediating autoinhibition of ACH release in the rat heart in vitro. Apparently, autoreceptors in the rat heart differ from those in the guinea pig ileum which belong to the smooth muscle or ileal type (1). Supported by DFG.
(1) Fuder et al., Eur.J.Pharmacol. 113:125, 1985.

24 Prejunctional inhibitory muscarine receptors modulating acetylcholine release in the guinea pig trachea: direct evidence by release experiments

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The presence of a p.ejunctional inhibitory muscarinic feed-back mechanism modulating the release of acetylcholine (ACh) from nerve terminals of the guinea pig trachea was investigated by a radioisotope method. Tracheal smooth muscle strips were obtained connecting crosswise with clips five pieces of four rings each and placed in Krebs-Henseleit solution that contained in addition propranolol, indomethacin and guanethidine. The preparations were preincubated with ^3H-choline and stimulated by electrical field stimulation by means of trains of 50 pulses at 10 Hz. Such a procedure improved

the content of tritiated ACh in the tissue. The field stimulation at 5 Hz (600 pulses) produced a release of tritium. Most of the induced outflow was found to be ^{3}H-ACh. Both tetrodotoxin treatment and calcium omission from the medium prevented such an evoked outflow. When two electrical stimulations (S_1 and S_2) were carried out at 5 Hz (600 pulses) at 30-min interval an S_2/S_1 ratio of 0.91 was found. Physostigmine reduced the evoked release of ^{3}H-ACh whereas atropine increased it in a concentration-dependent manner. Oxotremorine, carbachol and pilocarpine decreased the ^{3}H-ACh evoked outflow in a concentration-dependent fashion. The maximal inhibition in release was by about 90 %. These findings provided direct evidence that the trachea of the guinea pig is equipped with prejunctional muscarine receptors inhibiting the release of ACh from postganglionic nerves.

25 Differential effects of pertussis toxin (PTX) on the M₁- and M₂- receptor-mediated modulation of acetylcholine (ACH) release

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The cholinergic neurones of guinea pig myenteric plexus contain muscarine receptors whose stimulation causes either inhibition (M2) of the evoked, or increase (M1) of spontaneous release of ACh. We have investigated whether PTX-sensitive G-proteins are involved in the muscarine receptor-mediated modulation of ACH release. For comparison the influence of PTX on the effects of adenosine (A) were studied on isolated left guinea pig atria.

Guinea pigs were injected with PTX (60 μg/kg iv) and killed 3 days later. A and pilocarpine (P) had negative inotropic effects (EC50: 34 and 0.9 μM, respectively) which were significantly attenuated by PTX (EC50:330 and 12 μM). Myenteric plexus preparations prelabelled with [3H]choline were superfused with Tyrode solution and stimulated electrically (0.1 and 1 Hz). A (10 μM) reduced the evoked [3H]ACH release by 79 ± 3 % (N=8) in control experiments and only by 52 ± 4 % (N=5) in preparations from PTX-treated guinea pigs. Likewise, PTX treatment signficantly attenuated the inhibitory effect of 10 μM A on the electrically evoked contraction of the smooth muscle (77 ± 3 % vs 14 ± 2 %). The inhibitions of evoked [3H]ACH release by 0.1 μM oxotremorine (63 ± 2 %) and 30 μM P (51 ± 3 %) were not significantly affected by PTX pretreatment (65 ± 2 and 42 ± 6 %). However, the increase of [3H]ACh release by 30 μM P (4.48 ± 0.24 % of tritium tissue content, N=7) was significantly attenuated by PTX treatment (1.87 ± 0.34 %, N=13). It is concluded that PTX-sensitive G-proteins are involved in the receptor mechanisms coupled to soma-dendritic M1 receptors, but not in the M2-receptor mediated autoinhibition of ACH release.

26 Age-related changes in the muscarinic receptor autoregulation of acetylcholine release from rat brain

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Cholinergic function has been reported to change with age in the brain of several species. In addition, the status of muscarinic receptor sites may be altered in the normal aging process. Thus, we attempted to characterize the binding of muscarinic ligands to homogenates of 3 (young), 9 (middle-aged), and 27 (old) month-old rats and to study the regulation of endogenous acetylcholine (ACh) release by muscarinic autoreceptors from tissue slices. Our results demonstrate that while the

density of the total muscarinic receptor population, assessed using ^{3}H-QNB as ligand, is not altered in the aging rat brain, the density (Bmax) of muscarinic-M2 sites (quantified using ^{3}H-AF-DX 116) is significantly reduced in most brain areas (cortex, hippocampus, striatum, thalamus) by 9 months of age and further reduced by 27 months. Muscarinic-M1 sites, defined using ^{3}H-pirenzepine, were only modestly reduced in cortex and thalamus of 27 month old rats. In slices of cerebral cortex and hippocampus, where ^{3}H-AF-DX 116 binding was markedly reduced with age, the effect of exogenous AF-DX 116 to enhance ACh release was also significantly reduced. In slices from 3-month old rats, AF-DX 116 increased release by 40-50%; in the 9-month old rats, this effect ranged from 28-35%; in the old rats, this effect was only 11-25% of control (release in the absence of drug). However, in the striatum, where the density of muscarinic-M2 sites is reduced by 24% and 35% in the 9- and 27-month old rats, respectively, the AF-DX 116- induced increase in ACh release is similar to that in the 3-month old rat brain. In conclusion, it appears that the decrement in muscarinic-M2 receptor sites in the aging rat brain may be reflected in a change in autoreceptor function.

27 Effects of two different types of pyrethroids on subpopulations of muscarinic receptors in the neonatal mouse brain

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Pyrethroids are commonly divided into Type I compounds which lack an α-cyano substituent, and Type II compounds which contain a α-cyanophenoxybenzyl substituent. The main symptom of exposure to the former is tremor while choreoathetosis and salivation are the main symptoms of the latter. In this study ten-day-old NMRI mice were given either deltamethrin (Type II), bioallethrin (Type I) or the vehicle orally once daily for seven days. The doses used were as follows: deltamethrin, 0.71 and 1.2 mg/kg body wt.; bioallethrin, 0.72 and 72 mg/kg body wt.; and 20% fat emulsion vehicle, 10 ml/kg body wt. The mice were killed 24 hr after the last administration, and crude synaptosomal fractions (P2) were prepared from the cerebral cortex and hippocampus. At the lower dose, which did not cause any neurotoxic symptoms, both pyrethroid types caused a increase in the specific ^{3}H-QNB binding sites in the cerebral cortex. The proportions of high- and low affinity binding sites were assayed in a displacement study using ^{3}H-QNB/carbachol. Here deltamethrin caused an increase and decrease in the percentage of HA- and LA-binding sites, respectively, whereas the reverse was observed after bioallethrin treatment. The higher doses revealed typical symptoms of pyrethroid poisoning. At this dose deltamethrin affected the muscarinic receptors in the hippocampus, whereas bioallethrin had no apparent effect.

28 In-vivo regulation of CNS muscarinic receptor subtypes by chronic agonist and antagonist administration in rats

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Data from selective antagonists such as pirenzepine (P2) and AF-DX 116 (11-2-[[2-[(diethylamino) methyl]-1-piperidinyl] acetyl]-5,11-dihydro-6H-pyrido (2,3-b)(1,4)

benzodiazepine-6-one) in binding and functional assays lead to subtyping of muscarinic acetylcholine receptors (mAChR) (TIPS Suppl. III: 1988). Molecular cloning data show a family of five mAChR genes, each sharing the same proposed overall structure and extensive sequence homology. Our binding studies, in good agreement with in situ hybridization studies, imply three mAChR subtypes exist in CNS areas like corpus striatum (CS) and cerebral cortex (CC). [^{3}H]PZ, [^{3}H]AF-DX 116, [^{3}H](+)cismethyldioxolane ([^{3}H](+)CD), which can selectively label the super high affinity agonist site, and [^{3}H](-)quinuclidinylbenzilate ([^{3}H](-)QNB) binding were done as has been described. Membranes were incubated at 25°C, 1h or 2h, in 10mM sodium potassium phosphate buffer with 1mM magnesium in a 1ml volume. Effects on recognition sites for [^{3}H]labels were assayed after 14d chronic administration of these drugs (ip): atropine (AT)(10mg/kg/d), carbamylcholine (CAR)(1mg/kg/d) and an oxotremorine analog, BM-5 (5mg/kg/d; 10mg/kg/d). Dissociation constants (K_d) for [^{3}H](-)QNB (14-50pM), [^{3}H]PZ (2-12nM), [^{3}H](+)CD (1-5nM) and [^{3}H]AF-DX 116 (16-60nM) were similar. Regional studies revealed adaptation with altered estimates of receptor density (Bmax). AT increased [^{3}H](-)QNB binding in hippocampus>CS>CC, and [^{3}H]PZ binding was similar. In CC, CAR caused downregulation or subsensitivity (-36%, p<.05) by [^{3}H](-)QNB assay. BM-5 causes upregulation. BM-5 also raised CC [^{3}H]AF-DX 116 binding (151%, p<.05) but AT didn't alter CC [^{3}H](+)CD binding. Each subtype, and pre- and postsynaptic mAChR's appear to show differential and complex regulatory capabilities. Supported in part by FUMDNJ, BRSG & MH-43024.

29 Synaptosomal acetylcholine release is modulated by M_1 and M_2 mACh receptors

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The effect of McN-A-343 and that of oxotremorine on [^{3}H]ACh release and [^{3}H]choline transport were studied in cortical guinea-pig synaptosomes that had been preloaded with [^{3}H]choline after treatment with the irreversible ChE inhibitor DFP. Active release or uptake is the diffrence between total release or taken-up labeled compounds at 37°C and 0°C. McN-A-343, in a dose-dependent manner (10-1000 uM) enhanced the basal active release of [^{3}H]ACh, and atropine (1 µM) inhibited this process. McN-A-343 also enhanced the K$^+$-evoked (50 mM) active release. McN-A-343 at equivalent concentrations exerted an opposite effect on [^{3}H]choline transport. It depressed efflux, dose-dependently, both in the basal and in the stimulated states, and also depressed active [^{3}H]choline uptake to about one third at 1mM. These latter effects of McN-A-343 were not sensitive to atropine. The only effect of oxotremorine (10-1000 µM) in the present system was a decrease in the K$^+$-evoked release of [^{3}H]ACh. These findings are consistent with the following hypothesis: (1) presynaptic M1-ACh autoreceptors may functionally increase the outflow of ACh. (2) The excitatory response may be relatively more pronounced at resting state than in the stimulated state. This follows from the fact that the oxotremorine-sensitive inhibitory receptor is more responsive in the depolarized state. (3) The inhibitory receptor is most probably of the M2 subtye. (4) The activity of the choline transporter may be associated with the enhaced outflow of ACh, but in an opposite rrend.

30 Effects of THA on acetylcholinesterase and muscarinic receptors after single and repeated injection in rat

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Tetrahydroaminoacridine (THA), a potent acetylcholinesterase (AChE) inhibitor, has been shown to be active in the treatment of Alzheimer disease In roderts. Inhibition of AChE induced a down regulation of muscarinic receptors We have thus characterized the THA-induced AChE inhibition in vitro and the time course of ex vivo AChE inhibition after an acute injection of THA Acute and chronic effects of THA on muscarinic receptors were also studied.

AChE activity was determined by the method of Elmann Total muscarinic receptors and M_1 receptor subtypes were assayed using respectively [^{3}H]N-methylscopolamine (NMS) and [^{3}H]pirenzepine (PZ) saturation assays Low affinity PZ sites (M_2 receptor subtypes) were measured using [^{3}H]NMS / PZ competition

THA potently inhibited rat brain AChE in vitro with a K_i of 0.16 uM. In comparison, physostigmine (PHYS), another AChE inhibitor, was more potent (K_i = 0 014 uM)

After a single injection of THA (10 mg/kg i.p.), AChE activity was significantly inhibited during 5 hr with a maximum effect at 30 min. In comparison, at the dose of 0.4 mg/kg s c . the time course of PHYS-induced AChE inhibition was 2 hr.

THA displaced [^{3}H]PZ binding (10 nM) on hippocampal (M_1 receptor subtypes) and [^{3}H]NMS (0.3 nM) binding (M_2 receptor subtypes) on brainstem membranes with an IC$_{50}$ of 9 and 32 uM respectively.

After repeated injection of THA (start ng dose 2.5 mg/kg i p. with increments up to 10 mg/kg i p at 21 days), AChE activity of THA-treated rats was not significantly different from saline treated-rats. Forty hours after the end of the treatment, M_1 muscarinic receptor subtypes were significantly lowered in the cortex (-33 %) and in the striatum (-21 %) but not in the hippocampus The number of M_2 receptor subtypes was not significantly modified in these regions In all cases, Kd values remained unchanged

In conclusion, THA is a potent and long-acting inhibitor of rat brain AChE It is also more potent to inhibit brain AChE in vitro than to displace [^{3}H]PZ or [^{3}H]NMS binding. In addition, since cortical and striatal high affinity PZ sites seem to be generally post-synaptically located, our results suggest that selective down-regulation of M_1 receptors could be due to an over sensitivity of these receptors to chronic AChE inhibition However, it seems that hippocampal M_1 receptors could be differently regulated

31 Effects of parainfluenza virus on agonist affinity for muscarinic receptors in guinea-pig lung and heart

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By cleaving sialic acid from cell membranes, neuraminidase (NA) reduces the affinity of agonists for muscarinic receptors of the M2 but not of the M1 subtype (Biochem Biophys Res Commun, 1988, 150, 673). Because NA is present in large quantities on parainfluenza virus (PV), we examined the effect of PV on the affinity of agonists for muscarinic receptors in the heart (containing M2 receptors) and lungs (containing multiple receptor subtypes). The hearts and lungs of Dunkin Hartley guinea pigs were homogenized in 50mM Na+/K+ phosphate buffer and centrifuged at 1,000 x g for 10 min at 4°C. The supernate was centrifuged at 30,000 x g for 30 min The membrane pellets were resuspended in buffer (pH 6 0) to give whole organ weight/volume solutions of 7% for the heart and 20% for the lungs. Membrane solutions were incubated for 60 min at 37°C in the absence or precence of either NA (50 µU/ mg protein), PV containing 50 µU of NA/mg protein, or PV plus 1mM dehydro-deoxy-N-acetylneuraminic acid, an inhibitor of NA. Carbachol (CBC) competition against 0.2nM [3H]-quinuclidinyl benzilate (QNB) was carried out in buffer, pH 7.4.

In the lungs, CBC displaced QNB from two sites, (KD 0 2 uM (33%) and 13 µM (67%); data analyzed using LIGAND). However, in the presence of NA or PV the high affinity site was lost, and CBC displaced QNB from one site (KD 9.2 µM). In the heart, CBC displaced QNB from two sites, (KD 0.28 µM (42%) and 100 µM (58%)) Incubation with either NA or PV decreased the affinity of CBC for both sites in the heart and increased the percent of high affinity sites (KD 4 µM (70%) and 1400 µM (30%)). In both tissues the presence of NA inhibitor completely blocked PV-induced changes in CBC affinity. These results demonstrate that PV can decrease the

affinity of agonists for 33% of muscarinic receptors in the lung, and for all muscarinic receptors in the heart by the removal of sialic acid from muscarinic receptors. (Supported in part by NIH grants 1F32HL07691-01 CLN2, NS-25743, AG-07118, AG-00344, and the American Lung Association).

32 An endogenous factor induces heterogeniety of binding sites for selective muscarinic receptor antagonists in rat heart

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Pirenzepine (Pz) binds with low affinity to muscarinic receptors in purified membrane preparations of rat brain stem, while in crude membrane preparations Pz binds to 40% of the receptor population with high affinity (Biochem Biophys Res Commun: 1988, 157, 42). Because rat heart expresses only one subtype of muscarinic receptor (according to molecular biology and pharmacological criteria) with low affinity for Pz and high affinity for AF-DX 116, we examined whether heterogeniety of binding could also be demonstrated in this tissue. The hearts of Sprague-Dawley rats were homogenized in 50 mM Na+/K+ phosphate buffer, pH 7.4 before centrifugation at 1,000 x g for 10 min. at 4°C. The pellet was discarded and the crude homogenate was centrifuged at 30,000 x g for 30 min. The membrane pellet was resuspended in either buffer or supernate Final working solutions were 7% w/v of whole organ.

Both Pz and AF-DX 116 displaced 0.2 nM [3H]-quinuclidinyl benzilate (QNB) from one site in the pellet plus buffer (KD 270 nM, nH 0.95, and KD 64 nM, nH 0 92 respectively; data analyzed using LIGAND). However, when the supernate was added to the pellet, displacement curves for both antagonists best fit 2 site models. For Pz 27% of the sites had a KD of 4.8 nM, while the remaining 73% had a KD of 290 nM (nH 0 60). For AF-DX 116, 65% of the sites had a KD of 16 nM, while the remaining 35% had a KD of 460 nM (nH 0.69). The supernate did not contain muscarinic receptors as demonstrated by lack of saturable QNB binding. The addition of supernate to the pellet did not change either the Bmax or the KD of QNB for receptors in the membrane pellet. Our results indicate that an endogenous factor in the supernate produces high affinity binding of Pz and low affinity binding of AF-DX 116 to 30% of muscarinic receptors in the heart. Thus, while cardiac muscarinic receptors are coded for by a single mRNA, the resulting receptor protein might behave like a mixture of receptor subtypes in intact tissues. (Supported in part by NIH grants 1F32HL07691-01 CLN2, NS-25743, AG-07118, AG-00344).

33 Absence of a high-affinity binding site for acetylcholine in the aorta of the spontaneously hypertensive rat

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Tritiated acetylcholine (ACh) bound specifically to the muscarinic receptors in the aortae of the Wistar Kyoto (WKY), left renal artery stenosed WKY (LRAS-WKY) and spontaneously hypertensive rats (WKY). Both the endothelial and smooth muscle receptors in the WKY were heterogenous, each having a high and low affinity binding site for ACh. Similar patterns of receptor distribution were also observed in the aortae of the LRAS-WKY. However, there were significant reductions in the number of both the high (over 50%) and low (over 30 %) affinity binding sties in the endothelium of the LRAS-WKY. The endothelium of the spontaneously hypertensive rat (SHR) showed a complete absence of the high affinity binding site and a marked reduction (over 70 %) of the low affinity binding site. On the other hand the receptors in the smooth muscle were, with respect to their binding constants (Kd) and numbers (Bmax), similar to those of the LRAS-WKY and WKY.

The results obtained with the LRAS-WKY support our earlier observation that the aortae of these rats were hyporesponsive to the relaxant action of ACh (1). However, similar conclusion cannot be drawn for the SHR as we and other investigators have shown that the aortae of the SHR were not hyporesponsive to pharmacological doses of ACh.

1. Japan. J. Pharmacology. 39, 551–553, 1985.

34 Affinity of muscarinic antagonists for porcine coronary muscarinic receptors

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The existence of muscarinic receptors in coronary arteries and their involvement in muscle contraction is well established (Kalsner, J. Physiol. 358, 509 (1985)). While binding studies reveal the coexistence of binding sites of the M2(heart)- and the M3(submandibular gland)-subtype (v. Charldorp et al., Naunyn-Schmiedeberg's Arch. Pharmacol. 339(Suppl.), 328 (1989)) little is known about the functional subtype. Aim of this study is to compare pKi-values obtained in binding studies using the muscarinic antagonists atropine, pirenzepine, AF-DX 116, 4-DAMP, hexahydrosiladifenidol (HHSiD) and methoctramine with pA2-values of antagonism of metacholine induced contraction (table).

	pK_i	nH	pA_2
atropine	9.23	0.93	9.06
pirenzepine	6.87	0.86	6.98
AF-DX 116	6.38	0.75*	6.13
4-DAMP	8.79	0.89	9.20
HHSiD	7.83	0.83	7.94
methoctramine	6.67	0.74*	5.79

*significantly different from unity

[3H]-NMS binds to coronary artery preparations with a K_D of 135 pM and a B_{max} of 57 fmol/mg protein. Binding curves of AF-DX 116 and methoctramine are shallow with Hill-coefficients significantly different from unity. The binding data are in good agreement with those obtained in functional studies. A mixed population of M_2 and M_3 muscarinic receptor subtypes is present in porcine coronary arteries. However functional experiments do not support the contribution of the M_2-subtype to the contractile response.

35 Interaction of p-fluorohexahydrosiladifenidol (pFHHSiD) at muscarinic receptor subtypes *in vitro*

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pFHHSiD acts as a selective M_3 antagonist (Lambrecht et al. 1988; M_3 pA$_2$ = 7.8, M_1 pA$_2$ = 6.9, M_2 pA$_2$ = 6.0). We have assessed the affinity of pFHHSiD at a range of muscarinic receptors in isolated tissues. The methods used for each isolated preparation have been described previously (Eglen et al. 1988; O'Rourke and Van Houtte, 1987).

The mean ± S.E.M. pA$_2$ values and Schild slopes are shown below:

Preparation	Subtype	pA$_2$	Slope
G.P. Ileum	(M$_3$)	7.86 ± 0.04	1.0
G.P. Left Atria	(M$_2$)	6.14 ± 0.08	1.2
G.P. Trachea	(M$_3$)	7.10 ± 0.03	0.9
D. Saphenous Vein	(M$_3$)	7.08 ± 0.06	1.1
R. Aorta		7.68 ± 0.07	1.0
Rb Jugular Vein		7.74 ± 0.11	1.0

G.P.-guinea-pig; D-dog; R-rat; Rb-rabbit.

The pA₂ values obtained at the three subtypes were similar to values previously reported (Lambrecht et al. 1988). The similarity in values at receptors mediating relaxations of the vascular tissue indicate that M₃ receptors also mediate these responses. The reason for the anomolous value in the trachea is presently unclear but we do not conclude that it represents the presence of M₁ receptors (see also Eglen et al. 1988).

Eglen, R.M. et al., (1988) Br. J. Pharmac., 95, 1031-1038
Lambrecht, G et al., (1988) Eur. J. Pharmac., 152, 193-194.
O'Rourke, S.T. and Vanhoutte, P.M. (1987) J. Pharm. exp. Ther. 214, 64-67.

36 Characterization of muscarinic receptors mediating responses in canine femoral vein and artery *in vitro*

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Relaxations of the rat aorta are mediated by M₃ receptors (Eglen et al., 1988), whilst contractions of the canine saphenous vein are mediated by M₁ receptors (O'Rourke and Vanhoutte, 1987). We have determined the muscarinic receptor subtype in the femoral artery and vein mediating relaxations and contractions respectively.

Relaxant responses were determined in rings (2 mm) of artery precontracted with either phenylephrine (0.1 µM-pirenzepine, parafluorohexahydrosiladifen-idol; pFHHSiD) or U46619 (10 nM-methoctramine). Contractile responses only were determined in the vein. Carbachol was used as the agonist.

	Femoral Vein		Femoral Artery	
	pA₂	slope	pA₂	slope
Pirenzepine				
	7.86 (0.19)	0.98 (0.11)	6.26 (0.11)	1.07 (0.10)
pFHHSiD				
	7.11 (0.17)	1.08 (0.07)	7.90 (0.30)	0.75 *(0.11)
Methoctramine				
	6.07 (0.17)	1.17 (0.15)	6.05 (0.09)	1.01 (0.07)

*Significantly (p<0.05) different to unity. Values are mean (SEM).

The data were consistent with the presence of M₁ and M₃ receptors mediating responses of the femoral vein and femoral artery respectively. The femoral vein, like the saphenous vein, represents a novel tissue in which to bioassay M₁ receptor function.

Eglen et al., (1988) Br. J. Pharmac. 95, 1031-1038.

O'Rourke, S.T. and Vanhoutte, P.M. (1987) J. Pharm. exp. Ther. 241, 64-67.

37 Muscarinic abnormalities in hamster cardiomyopathy

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Maximum inhibition by carbachol of the steady-state, GTP-stimulated production of cyclic [³²P]AMP in washed left ventricular membranes was 50% in random-bred (RB) Syrian hamsters and 28% in cardiomyopathic (TO) hamsters. The inhibition was blocked fully by the muscarinic antagonist N-methylscopolamine (NMS) and

was characterized by Hill coefficients less than one, suggesting that multiple states or forms of the muscarinic receptor govern the response. Specific binding of [³H]NMS revealed a single class of sites of comparable capacity in each strain (120 - 200 pmol/g of protein). Guanylyl-imidodiphosphate (GMP-PNP, 0.1 mM) had no effect on K_D (TO, 520 ± 10 pM; RB, 700 ± 20 pM). Competition studies between carbachol and 1 nM [³H]NMS revealed three classes of sites for the agonist (K_H, K_M, K_L); upon the addition of GMP-PNP, there was a redistribution of receptors among the various classes, with an apparent increase in the value of K_M. Estimates of K_H, K_M (± GMP-PNP) and K_L were indistinguishable between the two strains; in contrast, the apparent redistribution of sites upon the addition of GMP-PNP was predominantly to the state of low affinity in RB tissue but to the state of medium affinity in TO tissue. Cardiomyopathy thus appears to reduce the nucleotide-promoted conversion to the state of lowest affinity; the implied change in the interaction between the receptor (R) and the inhibitory guanylyl nucleotide-binding protein (G) may underlie the reduced response. Mathematical simulations based upon an agonist- and nucleotide-regulated equilibrium between R and G (*i e.*, R + G⇌RG) suggest that any change in the propensity of RG to dissociate would be reflected in the dose-dependence of binding observed not only for carbachol but also for GMP-PNP. Contrary to this expectation, the disease was without effect on the potency of GMP-PNP in reversing the inhibition of [³H]NMS binding by 10 µM carbachol. It follows that the allosteric interaction between agonist and nucleotide may not involve dissociation of the RG complex or a subunit thereof. (Supported by the Medical Research Council of Canada and the Heart and Stroke Foundation of Ontario)

38 High affinity pirenzepine receptors in chicken atria are different from M₁ type

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Postjunctional muscarinic receptors mediating negative inotropic effects in chicken atria have been classified as being of the M1-type on the basis of a high pirenzepine affinity (Jerk et al., Br.J.Pharmacol. 93:357, 1988). In this study the affinities (pA₂-values) of a series of selective antagonists were determined in the chicken atria using APE as agonist and compared with those obtained in rabbit vas deferens (M1), guinea pig atria (M2) and ileum (M3).

	Chicken atria	M1	M2	M3
Pirenzepine	7.7	8.2	6.8	6.9
AF-DX 116	6.9	6.8	7.4	6.5
HHSiD*	7.0	7.9	6.5	7.9
4-DAMP	7.9	9.1	8.4	9.3
(S)-THP MeJ⁵	7.0	7.8	7.1	7.1
(R)-THP MeJ	8.5	10.6	9.0	9.2

*Hexahydro-sila-difenidol. ⁵Trihexyphenidyl methiodide.

All compounds tested proved to be competitive antagonists. The rank orders of antimuscarinic potencies were different among the four preparations. Thus, the postjunctional muscarinic receptors in chicken atria not only differed from M2 and M3, but clearly proved to be unlike M1 inspite of a high affinity of pirenzepine. This conclusion was supported by the finding that the M1-selective drugs McN-A-343 and 4-Cl-McN-A-343 were only poor partial agonists in chicken atria. These results suggest that a high affinity of pirenzepine is not sufficient to identify the M1-subtype unequivocally.
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39 Presynaptic autoreceptors in chicken heart exhibit M₁ characteristics

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In a previous study on perfused chicken hearts pirenzepine (Pz) at low concentrations (EC50 about 30 nM) enhanced the vagally induced release of acetylcholine (ACh), a result that was taken as evidence for the existence of presynaptic M1-receptors (Jack et al.,Br.J. Pharmacol, 93, 357, 1988). A similar conclusion was drawn from studies on cardiac pacemaker cells in man (Pitschner and Wellstein, Naunyn-Schmiedeberg's Arch Pharmacol 338: 207, 1988).- The present study further analyses the presynaptic high affinity Pz receptor in the chicken heart by measuring the enhancing effect of 6 antagonists on ³H-ACh-release evoked by field stimulation (5 Hz, 1 min; S₂/S₁).

Antagonist(Ant)	-log EC50	EC50Ant/EC50Atropine
Atropine	8 23	1.00
4-DAMP	8.36	1.47
Sila-hexocyclium	7.74	3.08
Pirenzepine	7.33	7.93
HHSiD	7.22	10.22
AF DX 116	6.49	54.91

It was found that the autoreceptor of the chicken heart exhibits, indeed, a high affinity for Pz as indicated by the high -log EC50 and the low potency ratio "Pz/atropine". In addition, the results obtained by the other antagonists are compatible with the characteristics of the M1-receptor as found in mammals.

40 Muscarinic binding sites in chicken heart and rabbit peripheral lung

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Muscarinic binding sites in peripheral tissues are generally classified as M2 or M3, i.e with low affinity for pirenzepine, but pirenzepine has relatively higher affinity for muscarinic binding sites in chick heart and rabbit peripheral lung (Brown et al., Mol Pharmacol., 27, 525, 1985; Bloom et al., JPET. 240, 51, 1937) We have characterized the muscarinic binding sites in these tissues and compared them with binding sites in rat tissues thought to represent M1, M2 and M3 receptors.

M1 sites were labelled with 0 5nM ³H-pirenzepine in rat cortex All other sites were labelled with ³H-NMS - M2 sites in rat heart and M3 sites in rat submandibular gland. After 2h incubation at 22°C in 20mM HEPES + 100mM NaCl, the assay was terminated with rapid filtration IC₅₀ values obtained with ³H-NMS were converted to Ki values using the equation Ki = IC₅₀/((L)/Kd+1)

-log Ki or -log Kd at various muscarinic binding sites

	M1	M2	M3	chicken heart	rabbit lung
³H-NMS	.	9 6	9 9	9.7	10.2
Pirenzepine	8.0	6.3	7 1	7.6	7.5
Himbacine	7.0	8.4	7.1	6.2	8.2
Methoctramine	7.3	8.4	6.6	7 9	7.7
4-DAMP	8.9	8.3	9.3	8.3	6.8
HSD	7.9	7.0	8.3	7.6	7.9
Secoverine	8.8	8.8	8.2	9.5	9.3

Mean pKi, n=3, SEM <0.1

The pKi values shown in the table indicate that the sites in CH and RL have a similar pharmacological profile. Pirenzepine has a relatively high affinity at these sites, but so do himbacine and methoctramine This pharmacological profile is different from that of M1, M2 or M3 receptors. Secoverine is slightly more potent at CH and RL than at M1. M2 or M3. Although the sites in CH and RL appear similar both 4-DAMP and ³H-NMS have lower affinities for CH than RL.

41 Pharmacological and biochemical characterization of muscarinic receptors in human airway smooth muscle

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Human airway smooth muscle tone is mainly controlled by the parasympathetic nervous system. Therefore, an increase in the muscarinic cholinergic responsiveness of the airways has been suggested to play a role in the pathogenesis of bronchial asthma. Moreover, muscarinic antagonists (anticholinergics) do have an important place in the treatment of chronic obstructive lung disease, especially chronic bronchitis and emphysema. The present study was undertaken to characterize the pharmacological subtype of muscarinic receptors mediating smooth muscle contraction in human central and peripheral airways and to investigate hydrolysis of phosphoinositides as a possible transduction mechanism in muscarinic receptor-mediated human peripheral airway smooth muscle contraction. Using smooth muscle strips from human tracheae obtained at autopsy and small bronchial rings (1-3 mm diameter) from human lung tissue obtained at surgery, it was found that methacholine-induced contraction was blocked by muscarinic antagonists (AF-DX 116, 4-DAMP methobromide, pirenzepine) with pA₂-values characteristic of glandular/smooth muscle type muscarinic receptors. In addition, ipratropium bromide showed high affinity (pA₂=9.25) and a slope of the Schild plot close to unity. Using small bronchial smooth muscle particles, it was established that methacholine caused a time- and concentration-dependent accumulation of inositol phosphates which was proportional to the amount of tissue protein and which could be blocked completely by 1 μM atropine. The lower pD₂-value for inositol phosphates production compared to contraction (5.0 vs. 5.9) indicates an important amount of receptor reserve, as has been found (to an even higher degree) in bovine tracheal smooth muscle (Meurs et al., 1988). In conclusion, the muscarinic receptors mediating human central and peripheral airway smooth muscle contraction have been characterized for the first time to be of the glandular/smooth muscle type as in bovine and guinea pig tracheal smooth muscle. This study is also the first demonstration that the human airway smooth muscle receptors are coupled to phosphoinositide metabolism, which opens a new possibility to investigate the biochemical basis of cholinergic hyperresponsiveness in bronchial asthma.

[1]Meurs et al., Eur. J. Pharmacol. 156: 271 (1988).

42 Differential labeling of presynaptic and postjunctional muscarinic receptors by ³H-N-methylscopolamine (NMS) in dog ileum

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The characteristics of atropine-sensitive binding of NMS have been investigated in preparations of isolated axonal varicosities from myenteric (HP), deep muscular (DMP) and submucosal (SMP) plexuses, and in purified smooth muscle plasma membranes from longitudinal (LM) and circular (CM) muscles of dog ileum. The content of neuronal and smooth muscle plasma membranes in membrane fractions has been evaluated by activities of ³H-saxitoxin binding and 5'-AMPase, as reported previously. In each membrane preparation NMS labeled apparently homogenous populations of non-interacting binding sites, as documented by the linearity of Scatchard plots and by Hill coefficients not significantly different from 1. However, the affinities of NMS for presynaptic (varicose) receptors were significantly different from the affinities for postjunctional (smooth muscle) receptors. In particular the Kd values for presynaptic receptors were 2-4 nM with Bmax values, in fmol/mg, 153±32 (HP), 195±44 (DMP), and 612±78 (SMP). Bmax means± D n=4). The Kd value for postjunctional receptors in both LM and CM was 16 nM with Bmax values 1991±181 (LM) and 2088±276 (CM). Carbachol and oxotremorine at concentrations required for near-maximal displacement of NMS binding caused approximately 25% inhibition of forskolin-stimulated adenylate cyclase activity in axonal varicosities of HP. It is concluded that a/ presynaptic and postjunctional muscarinic receptors in

dog ileum can be distinguished under conditions of in vitro labeling by their affinities for NMS b/ the muscarinic receptor-mediated presynaptic inhibition of neurotransmitter release in MP may involve the suppresion of cAMP-dependent pathway(s).

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Compound	-log EC 50		
	RVD-M1	RVD-M2[a]	GPI-M3
Arecoline	6.6	6.9	6.4
Carbachol	6.7	7.3	7.0
4-Cl-McN-A-343	6.7	5.5[b]	5.4
McN-A-343	6.0	4.8[b,c]	5.0[c]
Muscarine	7.1	7.7	6.6
Oxotremorine	7.7	7.8	7.6

a.Eltze, KJP 151, 205 (1988); b.M2-effects in GP-atria; c.intrinsic activity 0 %;

[3H]-NMS/pirenzepine competition experiments showed that 56% and 74% of the total muscarinic receptor population are of the M1-type in epididymal and prostatic ends of RVD, respectively. In conclusion, the prostatic end of RVD is a simple functional model to determine apparent M1-potencies of muscarinic agonists.

43 Onset and offset rate of M_1-receptor blockade by telenzepine in the rabbit isolated vas deferens

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Muscarinic M_1-receptor selective antagonists like telenzepine exert their inhibitory action on gastric acid secretion by blockade of M_1-receptors located in the vagal pathway to the parietal cells. In the rabbit isolated vas deferens, stimulation of M_1-receptors by McN-A-343 inhibits electrically induced twitch contractions susceptible to competitive blockade by antimuscarinics (pA$_2$ values). This model was used to study the kinetic parameters of antimuscarinics on this receptor subtype. Inhibition of twitch contractions induced by 10^{-6} M McN-A-343 (EC$_{70}$) was reversed by antimuscarinics (at concentrations 10-fold higher than pA$_2$); the antagonist was then displaced by 3×10^{-5} M McN-A-343 (30-fold EC$_{70}$). The following t$_{1/2}$ values of onset and offset rates at 31°C were observed:

Drug	pA$_2$	t$_{1/2}$ (min), at -log M	
		Onset	Offset
Telenzepine	8.66	24.5	162.0	7.86
Pirenzepine	7.79	3.0	13.0	6.79
Nuvenzepine	7.74	3.7	2.7	6.74
BTM-1086	8.44	14.0	5.3	7.44
Atropine	9.46	13.5	4.2	8.46
Dicyclomine	7.70	9.0	1.5	6.70

Telenzepine exhibited the slowest rate of onset and offset among the muscarinic receptor antagonists tested. For telenzepine and pirenzepine onset was faster than offset rate; the converse was true for the other antagonists. Thus, the kinetics of telenzepine observed in vitro can partially explain its long duration of action in vivo. Ganglionic M_1-receptors which bind telenzepine will not respond rapidly to high concentrations of neuronally released acetylcholine; the receptor is thus protected from vagal overstimulation.

44 M_1-Potencies of muscarinic agonists in rabbit isolated vas deferens

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The rabbit vas deferens (RVD) has prejunctional inhibitory M1- and postjunctional facilitatory M2-receptors. In this study, M1-potencies of muscarinic agonists were determined in isolated preparations of RVD close to the prostate. The inhibitory effects obtained were antagonized by pirenzepine (20 - 50 nM). The results were compared with those obtained at M2-receptors in epididymal segments of RVD and at M3-receptors in guinea-pig ileum (GPI). The rank order of M1-potency was different from that found at M2- and M3-receptors.

45 Functional determination of McN-A-343 affinity for M_1 mAChRs

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Recent evidence showed that McN-A-343 elicits relaxation of isolated rat duodenum and inhibition of twitch contractions in isolated rabbit vas deferens by selectively stimulating M1 mAChRs (Micheletti et al., 1988, Eltze, 1988). Aim of the present study was to investigate whether affinity or intrinsic efficacy were responsible for the observed selectivity of McN-A-343. Affinity was determined by fractional inactivation of receptors performed with the irreversible muscarinic antagonist propylbenzylcholine mustard (PrBCM). After constructing control concentration-response curves, duodenal preparations were exposed to 1 μM PrBCM for 30 min. The tissue was washed for 60 min before repeating agonist stimulation. Vasa deferentia were exposed to 30 nM PrBCM for 20 min. From a reciprocal plot of agonist equiactive concentrations the value of pKA was estimated. McN-A-343 relaxed rat duodenum with a potency (-log EC50) of 6.31 ± 0.08 and affinity (pKA) of 4.68 ± 0.10 (n=6). In the same preparation, McN-A-343 antagonized ACh-induced contraction with a pKB of 4.97 ± C.14 (n=13). In the vas deferens, the estimated EC50 and pKA values were. 5.97 ± 0.09 and 5.17 ± 0.15 (n=8), respectively. Thus, affinity of McN-A-343 for the functional M1 receptors at which it acts as agonist is in the range of its affinities as antagonist. Furthermore, EC50 values lower than KA indicate a substantial receptor reserve. The latter or a more efficient coupling of M1 receptor to transduction mechanisms seems therefore to underlie McN-A-343 selectivity.

R Micheletti et al., J.P.E.T., 1988, 244, 680.
M. Eltze, Eur. J Pharmacol., 1988, 151, 205.

46 Characterization of muscarinic receptors in rat kidney

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Muscarinic receptors in mammalian kidney seem to be involved in diuresis. The presence of these receptors in rat kidney has been reported by radioligand binding only recently. However, no detailed characterization has been taken place. In this study we labeled the muscarinic receptors in rat kidney cortex homogenate by 3H-1-QNB at 37°C. The dissociation constant, K_D, = 0.058 ± 0.004 nM

and the receptor density, B_{max}, = 1.25 ± 0.07 pmol/g tissue (mean ± SE, n = 7). ^{3}H-NMS binding showed a similar B_{max}. Binding was reversible and the dissociation constant calculated from the ratio of the rate constants amounted to 0.019 nM. ^{3}H-1-QNB binding was inhibited by the enantiomers of benzetimide with a stereoselectivity ratio of 8,000. The inhibition of ^{3}H-1-QNB binding was investigated with the subtype selective antagonists pirenzepine (K_T = 226 ± 24 nM), AF-DX 116 (K_T = 1451 ± 72 nM), methoctramine (K_i = 1669 ± 33 nM) and hexahydrosila ditenidol (K_T = 40.4 ± 1.8 nM) characterizing the muscarinic receptor in the rat kidney as a M3 type. Inhibition of ^{3}H-1-QNB binding by the agonists methylfurtrethor um, arecoline, isoarecoline and arecaidine propargyl ester, displayed monophasic inhibition curves. With cis-2-methyl-4-dimethylaminoethyl-1,3-dioxolane methiodide in two out of four experiments, a small (11%) population of high affinity agonist sites could be detected Including inhibitors of proteolysis did not affect the agonist inhibition. The potassium sparing diuretic amiloride inhibited ^{3}H-1-QNB binding in a competitive way (K_i = 36 ± 5 uM). Inhibition appeared to be dependent on NaCl concentration. Autoradiographic analysis of the binding revealed the presence of specific ^{3}H-1-QNB labeling in glomeruli. No specific labeling could be observed in tubuli.

47 Histamine-liberating cells in the mouse isolated stomach may bear muscarinic M_1-receptors

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Acid secretion induced in the mouse stomach in vitro by the supposed M_1-selective muscarinic agonist McN-A-343 is inhibited by antimuscarinics and H_2-receptor antagonists, and is prevented by prior histamine depletion by compound 48/80. TTX has no influence. This suggests muscarine receptors to be located on histamine-liberating paracrine cells. In order to characterize the receptor type, 9 muscarinic antagonists with different selectivities were used at a fixed concentration of 1 umol/l to inhibit acid secretion stimulated by 10 umol/l McN-A-343. Quasi "concentration"-inhibition curves resulted from plotting percentages inhibition vs the concentration of 1 umol/l expressed as multiples of K_i values at M_1, M_2 and M_3 receptors. K_i values were determined in binding experiments with guineapig cortical (^{3}H-telenzepine, M_1), atrial (^{3}H-NMS, M_2) and salivary gland (^{3}H-NMS, M_3) membranes. By fitting of the 3 sets of data with a single receptor model this method of evaluation (Kromer, unpublished) allows discrimination between receptor types involved without the time-consuming task of determining pA$_2$ values.

Our data gave the best fit when calculating the curve on the basis of binding affinities to the M_1 receptor. No fit at all or a much worse fit was found on the basis of binding affinities at the M_2 and M_3 receptor, respectively. The present data demonstrate for the first time that histamine-liberating cells in the mouse stomach probably bear functional M_1-type muscarine receptors.

48 Muscarinic receptor binding in rabbit superior cervical ganglia

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The rabbit superior cervical ganglion is a neuronal preparation often used for investigating muscarinic receptor-mediated slow synaptic potentials. The slow excitatory postsynaptic potential (sEPSP) is elicited via activation of M1-receptors and the slow inhibitory postsynaptic potential (sIPSP) via M2-receptors.

Radioligand binding was performed on membrane fractions, isolated from over 100 ganglia. 3H-NMS binding was saturable; non-specific binding was less than 30% of total binding. Scatchard analysis indicated a single binding site (K_D=0.25 nM; Bmax=364 fmol/mg protein). In competition experiments with telenzepine (TZP), pirenzepine (PZP) and AF-DX 116, displacement curves with a shallow slope were obtained; Hill coefficients were less than unity. Fitting the data with a two site model revealed that about 50% of the sites displayed high affinity for TZP and PZP (M1-receptors); AF-DX 116 bound preferentially to the other 50% (M2-receptors). Dissociation constants (-log K_T) at the M1 and M2 sites were: TZP, 9.03 and 7.75; PZP, 7.73 and 6.23; AF-DX 116, 6.05 and 7.48 respectively. 3H-TZP binding revealed a single binding site for the (+)enantiomer (K_D=0.64 nM; Bmax=257 fmol/mg protein). Dissociation constants (-log K_T) at this site were: TZP, 9.02; PZP, 7.88 and AF-DX 116, 6.09.

The results demonstrate the existence of two distinct muscarinic receptor sites in these neurones.

49 [^{3}H]-(±)-Telenzepine selectively labels muscarinic M_1-receptors in calf superior cervical ganglia

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Sympathetic ganglia possess excitatory M1- and inhibitory M2-receptors. To study the affinities of antimuscarinic drugs at M1-receptors a simple binding assay has been developed by using the M1-selective ligand [^{3}H]-(±)-telenzepine (Tz) (Byk Gulden, Konstanz, FRG) and a purified preparation of calf superior cervical ganglia (CSCG). Muscarinic receptor binding was examined in 1000 x g supernatants (20% of original wet weight).These were incubated for 120 minutes at 37°C in Tris buffer(pH 7,4). The reaction was terminated by rapid filtration. In this preparation [^{3}H]-(±)-Tz (0.05 - 8 nM) bound to a homogeneous class of binding sites with high affinity (corrected K_d-value for the active (+)-enantiomer: 0 56 nM) Equilibrium was reached within 90-120 minutes. Nonspecific binding was determined by 10 µM atropine and amounted to 16% (maximal value) in saturation experiments and 4% in competition experiments (0.8 nM [^{3}H]-(±)-Tz). The affinities of reference antimuscarinic drugs obtained in competition experiments in CSCG were very similar to those found in functional studies at M1-receptors in rabbit vas deferens (RVD)(Eltze, NSAP 337, Suppl. R91, 1988).

	-log K_i (CSCG)	pA$_2$ (RVD-M1)
Pirenzepine	7.77	7.79
Methoctramine	6.63	6.85
HHSiD	7.65	7.92

In conclusion, CSCG homogenates are well suited to determine M1-receptor affinities of antimuscarinic drugs using [^{3}H]-(±)-Tz as radioligand.

50 Differential responses upon M₁ muscarinic receptor stimulation by various agonists

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At present, a discrepancy concerning possible diversity of central muscarinic (M1) receptors exist. Whereas Lambrecht et al.* according to different effects of the antagonists hexahydrodifenidol and hexocyclium published that the M1 receptors in rat ganglia and hippocampal slices are different, no difference was found by Field et al.*. Differential responses as well are found, however, when ganglionic or hippocampal M1 receptors are stimulated by various muscarinic agonists. The compounds muscarine, pilocarpine, McN-A-343 and its 4-chloro-analog acted as full M1 agonists, whereas oxotremorine displayed partial agonistic behaviour. Conversely, McN-A-343 and its 4-chloro-analog elicited no responses in rat hippocampal slices, whereas muscarine, pilocarpine and oxotremorine acted as full agonists. The differential activity of M1 agonists in rat ganglia and hippocampal slices cannot be explained by a difference in the number of M1-receptors in both tissues. It remains to establish whether a difference in the M1 receptor protein or a difference in the receptor to second messenger coupling is responsible for the diverging agonist activity in both tissues.

Lambrecht et al., TIPS suppl., abstr. 22, 1988.
Field et al., Soc. Neurosci. abstr. 435.11, 1988.

51 Mediation of phosphinositide metabolism in rat cerebal cortex by M₁ and M₃ muscarinic receptors

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The stimulation of phosphoinositide (PI) metabolism by muscarinic agonists was studied in cell aggregates from rat cerebral cortex, prelabeled with [3H]m-inositol. Muscarinic agonists assayed in the presence of LiCl produced a 4-fold scimulation with the rank order of potencies of: acetylcholine, oxotremorine-M > muscarine > carbamylcholine (CBC), methacholine (MET). With the exception of MET, the PI response to a fixed concentration of the muscarinic agonists was inhibited by pirenzepine (PIR) in a biphasic manner, with 62 to 75% of the sites showing high affinity (pKi~8.1) and the remaining sites of lower affinity (pKi~6.6). PIR inhibited MET effect with a pKi of 7.9. Telenzepine (TEL) also showed a biphasic inhibition of CBC effects with pKi of 9.4 (74 % of the sites) and 7.3. The rest of the antagonists assayed inhibited the effects of CBC in a monophasic manner: atropine (pKi~9.0), 4-DAMP (pKi~8.6), hexahydro-sila-difenidol (pKi~7.5), AF-DX 116 (pKi~6.6). PIR and TEL displaced [3H]NMS from multiple sites (50% high affinity) and [3H]TEL from a single binding site with high affinity. AF-DX 116 displaced 55% of [3H]NMS with low affinity, similar to that obtained by competition with [3H]TEL (pKi~5.9). Alkylation of muscarinic receptors with 30 nM propylbenzylcholine mustard in the presence of 100 nM PIR yielded a preparation with only high affinity sites for PIR both in PI response and [3H]NMS binding. Based on the multiphasic inhibition of the PI response by PIR and TEL, our data indicate the involvement of another muscarinic receptor besides the M₁ subtype. Furthermore, the rank order of potencies of the muscarinic antagonists provides strong evidence for the involvement of a glandular M₃ subtype in the activation of PI metabolism in rat brain.

52 PC12 (phaeochromocytoma) cells express a muscarinic receptor with high affinity for telerepine

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Functional and binding experiments in PC12 cells indicate that this neuronal cell line is endowed with M1- and probably also M3-receptors. Carbachol caused an atropine-sensitive release of 3H-noradrenaline (3H-NA) from preloaded cells which was inhibited with high potency by the M1-selective muscarinic antagonist telenzepine (TZ), with intermediate potency by pirenzepine (PZ), and with low potency by the M2-selective antagonist AF-DX 116 (see Table). Inhibition of 3H-NMS binding to PC12 membranes by these antagonists showed the same inhibitory profile; in addition, the affinities of several muscarinic antagonists to the PC12 receptor was similar to that obtained at M1-receptors in guinea pig membranes labelled with either 3H-TZ (M1) or 3H-NMS (M2 and M3). Saturation binding of 3H-TZ to PC12 membranes revealed a Kd of 0.55 nmol/l and a Bmax of 766 fmol/mg protein. Dissociation of 3H-TZ (bound to PC12 membranes at a high concentration) was biphasic indicating binding of 3H-TZ to M1-sites (80%) and probably M3-sites (20%).

Compound	PC12 3H-NA release pA2	PC12 3H-NMS binding pKi	guinea pig membranes M1 cortex pKi	M2 heart pKi	M3 gland pKi
Atropine	8.62	8.52	8.97	8.55	8.75
Telenzepine	8.82	8.74	9.00	7.64	8.10
4-DAMP	----	8.46	8.79	7.94	8.70
Dicyclomine	----	9.00	8.21	6.97	7.83
Pirenzepine	7.00	7.51	7.72	6.43	6.72
HHS1D	7.49	7.29	7.63	6.80	7.79
AF-DX 116	5.74	5.77	6.28	7.23	5.88

## 53 Novel selective muscarinic agonists

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A series of analogues of the muscarinic agonist BM5 were synthesized using the functionalized congener approach to drug design (Jacobson et al., Mol. Pharmacol. 29 126, 1986). These compounds were evaluated for subtype-specific agonist activity at the second messenger level in a series of cell lines that each express a single muscarinic receptor subtype. These cells include CHO cells transfected with m1 receptors, rat heart membranes that express only m2 receptors, SK-N-SH human neuroblastoma cells that express predominantly m3 receptors and NG108-15 neuroblastoma x glioma cells that express only m4 receptors. The parent compound, BM5, was found to stimulate only the adenylate cyclase-coupled muscarinic receptors, subtypes m2 and m4. It behaved as an antagonist at the PI turnover coupled receptors, subtypes m1 and m3. Therefore, BM5 is a subtype-selective muscarinic agonist. Since BM5 has been described as a post-synaptic agonist and a pre-synaptic antagonist (Nordstrom et al., Mol. Pharmacol. 24 1-5, 1983), these studies indicate that post-synaptic muscarinic receptors are adenylate cyclase coupled, whereas pre-synaptic muscarinic receptors are coupled to PI turnover. Like BM5, several of the novel compounds were also subtype-selective partial agonists in that they stimulated only the m2 and m4 subtypes and were inactive as agonists at m1 or m3 receptor subtypes. These compounds are being evaluated further for their potential in treating the cognitive deficits of Alzheimers disease.

54 Tetrahydropyridyl-oxadiazoles: ligands used to map the muscarinic receptor

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Fully rigid ligands may not have the required
flexibility[1] in order to evoke the conformational
change of the receptor protein necessary for a full
agonist response, and are at best weak partial
agonists. We have synthesised a series of semi-rigid
ligands based on arecoline in order to explore the
distinct binding characteristics for both the high and
low affinity states of the muscarinic receptor These
compounds differ markedly both in their relative
affinity and efficacy as measured by our well described
biochemical assay, the NMS/OXO-M binding assay[1]. The
oxadiazole 1 was synthesised from arecoline[2] and shown
to have greater affinity than the parent. Molecular
mechanics calculations have shown that the des-N-methyl
analogue 2 possesses a lower ring inversion barrier than
1 thus has greater flexibility within the receptor.
Measurement of binding energies, when compared to
computed average binding energies[3], revealed that 2 is
well matched particularly for the high affinity binding
site and this is reflected in a correspondingly higher
level of efficacy. In contrast alkylation on the 5 or
6-positions of the tetrahydropyridyl ring creates a more
rigid framework relative to 2. This provides ligands
which do not fully utilise their available functional
groups in efficient binding possibly because of a
combination of steric and conformational factors.
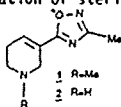
1. J. Med. Chem., (1988), 31, 486.
2. J.C.S. Chem Commun., (1988), 1618.
3. J. Med. Chem, (1984), 27, 1648.

55 5-Hydroxyquinuclidine oxadiazoles: stereochemical probes for the agonist binding state of the muscarinic receptor

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The oxadiazole 1, recently described[1] as a potent
muscarinic agonist, offers an ideal opportunity to probe
the steric requirements of the muscarinic receptor by
introducing substituents into the rigid quinuclidine
skeleton. Modification of the 5-position was studied,
since this site is remote from the primary pharmacophore.
Insertion of a hydroxyl group was chosen to search for
additional hydrogen bonding interactions over those
already known to be involved.
All four diastereoisomers of the 5-hydroxy quinuclidine
oxadiazole 2 were synthesised by a route involving
Dieckmann cyclisation of a differentially protected
piperidine diester. Three of the isomers had
considerably reduced affinity for the receptor c...1,
but one isomer, 2a, displayed greatly enhanced efficacy
as measured by the previously reported NMS/OXO-M
ratio[2,3].Thus one particular orientation of hydroxyl
favours agonist binding; structural properties of 2 will
be discussed in detail.

1. J. Saunders et al, J.C.S. Chem. Comm., 1988, 1618.
2. J. Saunders et al, J. Med. Chem., 1988, 31, 486.
3 S Freedman et al, Br. J. Pharmacol., 1988, 93, 437
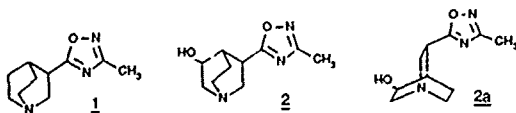
## 56 Heterocyclic muscarinic agonists as experimental tools and potential therapeutic agents

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Bioisosteric modifications of arecoline, norarecoline and
isoarecoline have led to a variety of CNS-active muscari-
nic agents. The aim of this work is to develop therapeu-
tically useful muscarinic agents with optimally balanced
partial agonist character and receptor selectivity and
with pharmacokinetic properties acceptable for use in the
human clinic. The partial agonist or antagonist profiles
of the compounds at M-1 or M-2 receptors have been esti-
mated using binding and functional in vitro and in vivo
tests.

O,5-Di-Me-THPO is an isoxazole bioisostere of areco-
line, and O,6-di-Me-THIP is similarly related to isoar -
coline. Whereas arecoline and isoarecoline have very si-
milar muscarinic agonist profiles, those of their respec-
tive isoxazole bioisosteres are quite different. Molecu-
lar modelling techniques have been used to elucidate the
conformational and electronic characteristics of this
class of muscarinic agonists. These studies have led to
muscarinic agonists with potential clinical usefulness
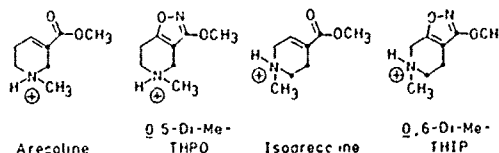
P Krogsgaard-Larsen, E. Falch, B Hansen and F G Jor-
gensen Drugs of the Future (1989) In press

57 Interaction of quaternary anticholinergics with muscarinic receptors in bovine, guinea pig and human airway smooth muscle

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Quaternary muscarinic antagonists (anticholinergics) have been shown to interact
with two populations of binding sites in bovine and rat brain, in contrast to
their tertiary analogues. Because we found this binding heterogeneity also in
bovine tracheal smooth muscle membranes, we tested these compounds in contraction
experiments on denuded bovine tracheal smooth muscle strips to establish whether
this heterogeneous binding behaviour has functional consequences. As reported
(Roffel et al., N.S. Arch. Pharmacol, 338 Suppl : R62 /1988) ipratropium (IPRA)
as well as N-methylscopolamine (NMS) and oxyphenonium (OX) gave Schild plots with
slopes significantly greater than unity when blocking methacholine-induced smooth
muscle contraction, using a 30 min equilibration period for the antagonists. By
contrast, atropine (ATR) and scopolamine on bovine trachea and IPRA on guinea pig
trachea gave slopes not significantly different from one. In the present study
these data were extended using 4-DAMP methobromide (pA2=9.0, slope=1.1) and
methyldeptropine (MD) (slope=2.0, P 0.005) on bovine tracheal smooth muscle, MD
on guinea pig tracheal smooth muscle (pA2=9.8, slope=1.1) and IPRA on human small
bronchial smooth muscle (pA2=9.2, slope=1.0). The results suggest that (i) high
Schild plot slopes occur in bovine but not in guinea pig and human airway smooth
muscle preparations, (ii) there is no relation between high Schild plot slopes
and heterogeneous binding behaviour, since MD does not exhibit the latter in
bovine brain membranes, and (iii) the occurrence of high Schild plot slopes is not
dependent on quaternary character and high affinity alone. To investigate the
possibility of lack of equilibrium at lower antagonist concentrations, IPRA and
MD were tested using a 3 hr equilibration period. Although the slopes decreased
for both compounds (to 1.5) they were still significantly greater than unity.
Moreover, in one experiment using a 5 hr equilibration period IPRA still gave a
slope of 1.5. We also tested the effect of the atropinesterase-inhibitor methyl
butyrate: both IPRA and MD still yielded Schild slopes greater than unity in
these experiments (1.9 and 2.1 respectively). However, using a combination of

ATR+IPRA and ATR+OX we found an overadditive right-shift of the methacholine dose-response curves in bovine but not in guinea pig trachea. We conclude that a positive cooperative interaction but not lack of equilibrium is responsible for the high slopes in the Schild plots of IPRA, NMS, OX and MD in bovine tracheal smooth muscle. Supported by the Netherlands Asthma Foundation.

58 AQ-RA 741- a new cardioselective (M₂) muscarinic antogonist

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Recently compound AF-DX 116 (I) has been characterized as the first cardioselective muscarinic receptor antagonist. In order to further elucidate the structural requirements for M_2 selectivity, a series of AF-DX 116 analogs has been prepared as indicated in formula II.
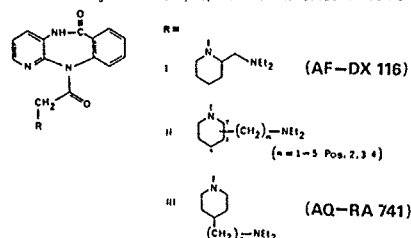
The biological profile of the new compounds has been assessed by receptor binding studies [K_i-values: Cerebral cortex (M_1), heart (M_2), submandibular gland (M_3)] and functional tests (pA_2-values: heart and ileum).
The results show that all compounds exhibit a selectivity profile analogous to that of AF-DX 116 with varying degrees of selectivity and activity.
Most favourable results have been obtained with compounds carrying the diethylaminoalkyl group in position 4 of the piperidine ring. Compound AQ-RA 741 (III) e.g., shows a 30 fold higher affinity for M_2 compared to M_3 binding sites. Functional studies revealed an even higher discrimination (90 fold) between atrial and ileal muscarinic receptors.
Thus, concerning cardiac vs. glandular and cardiac v.. smooth muscle selectivity AQ-RA 741 represents one of the most selective M_2 receptor antagonists known so far. In addition AQ-RA 741 is 10 times more active than AF-DX 116.

59 AQ-RA 741 and UH-AH 37 - two pirenzepine analogues with an opposite selectivity for atrial and ileal muscarinic receptors

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We investigated the selectivity of the novel muscarinic antagonists AQ-RA 741 (11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-on hydrochlorid) and UH-AH 37 (6-chloro-5,10-dihy-dro-5-[(1-methyl-4-piperidinyl)-acetyl]-11H-dibenzo-[b,e][1,4]diazepine-11-one hydrochloride) for atrial and ileal muscarinic receptors, respectively. Functional studies were performed using spontaneously beating right atria and ileal longitudinal muscle strips from guinea pigs. Concentration-response curves were constructed by the cumulative addition of methacholine in the absence or presence of antagonist. Pirenzepine (PZ) was not able to discriminate between the two tissues. However AQ-RA 741 showed a marked cardioselectivity (87-fold). An opposite ileal selectivity was observed for UH-AH 37 (14-fold). All three

compounds proved to be competitive antagonists. (see below)

	pA₂ heart	slope	pA₂ ileum	slope
PZ	6.64 (26)	1.01	6.80 (25)	0.98
AQ-RA 741	8.49 (32)	1.02	6.55 (28)	0.94
UH-AH 37	7.09 (30)	1.05	8.32 (38)	1.08

( ) number of data points
Comparison of the selectivity of AQ-RA 741, AF-DX 116 and methoctramine revealed that the cardioselectivity of AQ-RA 741 is comparable or even more pronounced. The same holds true for the ileal selectivity of UH-AH 37 in comparison to 4-DAMP or HHSiD.

60 Hexocyclium derivatives with high selectivity for smooth muscle muscarinic receptors

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Among compounds able to differentiate between atrial and smooth muscle mAChRs, those with greater affinity for muscular receptors are only moderately selective. Since previous evidence showed that introduction of a guanidino moiety as cationic head can enhance pirenzepine selectivity (Cereda et al., this meeting), the influence of different amidino substitutions on the discriminative properties of a smooth muscle selective antagonist was investigated. Modifications were brought on hexocyclium onium head (H). Formamidino-, acetamidino-, and guanidino derivatives of H (compounds F, A and G, respectively) were evaluated for antagonism of guinea pig ileal contraction and atrial negative inotropism elicited by bethanechol. Affinity (pA₂) was estimated from Schild regression analysis. Compounds yielded pA₂ of 9.01 ± 0.28 (H), 8.56 ± 0.13 (F), 7.68 ± 0.14 (A), 7.40 ± 0.15 (G) at the ileal receptor, and 8.32 ± 0.27 (H), 6.39 ± 0.09 (F), 5.74 ± 0.05 (A), 5.65 ± 0.07 (G) at the cardiac M₂ receptor. Although the substitutions performed were generally detrimental to compounds affinity, the pA₂ values at the two receptors were reduced by a significantly different extent. Thus, affinity for cardiac receptors was drastically affected, with a minimum decrease of 85 folds and maximum of ≈ 470; conversely, changes in ileal pA₂ were much lower, ranging from 2 8 to 41 folds The overall consequence of such a different effect on affinities is that hexocyclium ability to distinguish between receptors results greatly augmented In particular, F discriminates by over 2 orders of magnitude, possessing the greatest selectivity for smooth muscle receptors so far described

61 Antimuscarinic potencies of olefinic analogues related to hexahydro-difenidol at three muscarinic receptor subtypes

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Affinity profiles of olefinic analogues related to hexahydro-difenidol (1A) were investigated at three muscarinic receptor subtypes Antagonist potencies were determined in rabbit field-stimulated vas deferens (M1), guinea-pig electrically-driven left atrium (M2) and guinea-pig ileal longitudinal muscle (M3) pA2 values obtained from Arunlakshana-

Schild plots are listed below

	A/B
1A/1B	CH_2-CH_2
2A/2B	$CH=CH$ (cis)
3A/3B	$CH=CH$ (trans)

	M1	M2	M3			M1	M2	M3
1A[a]	78	67	8.0		1B	7.4	6.8	7.0
2A	7.1	65	7.5		2B	69	68	64
3A	88	7.5	8.1		3B	7.9	7.4	77

[a]Hexahydro-difenidol

The results indicate that the selectivity profiles of the olefinic analogues were quantitatively and qualitatively different from that of the parent compounds (1A/1B)

In both series, the trans analogues (3A/3B) were more potent than the cis compounds (2A/2B) at all subtypes. In particular, the trans compound 3A showed the highest affinity for the M1 receptor. Its affinities for the M3- and M2-receptors were 5- and 22-fold lower, respectively

62 Pharmacophore model for antimuscarinic agents related to difenidol and siladifenidol - a molecular modeling study

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In continuation of previous studies (1,2) antimuscarinic Si/C pairs of the (sila-)procyclidine, (sila-)difenidol, and (sila-)hexocyclium types were investigated using molecular mechanics and semi-empirical quantum chemical methods.

For a group of 25 analog pairs a potentially bioactive conformation has been established with the aid of a multifit procedure (see Fig. 1).

From the correlation of electron densities at potentially pharmacophoric partial structures with affinities to ileal M3 receptors the three binding sites previously postulated could be supported.

Fig 1

Fig. 2
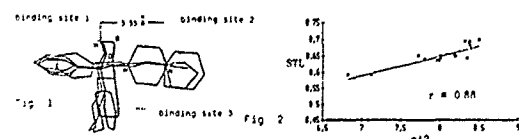
Calculations of electron densities were performed for the protonated molecules complexed with a negatively charged binding-site model. The molecular electrostatic potentials (MEPs) of all sila-compounds carrying a cyclohexyl ring show a distinct negative region around the pole of this ring system which does not occur in the charged compounds. We believe that this negative potential reduces receptor affinity when this effect is taken into account a strong correlation between theoretical data (STL) and M3 receptor affinities (pA2) is observed (see Fig. 2).

1) Busch, Th.; Dissertation Freie Universität Berlin (1987).
2) Lambrecht, G. et al. in Trends in Med. Chem. "88",
   Goot et al. (Eds.), Elsevier, Amsterdam 1989, 265.

63 Potent and selective mAChR antagonists with amidine cationic heads

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Amidino moieties resemble tertiary amino or quaternary ammonium groups according to basic strength, ionization properties, nitrogen orbitals hybridization pattern and size. Differences however exist in spatial arrangement, hydrogen bond forming capacity and possible modes of interaction with receptors. For the latter reasons, variations in the affinity for mAChR subtypes may be expected to occurr in amidino-substituted compounds. This hypothesis was tested by introducing an amidino moiety as cationic head in the structure of a non-selective antagonist, atropine (ATR), and of an M1 selective compound. pirenzepine (PZ). Affinity (KB) of compounds for M1 and M2 receptors was investigated in functional in vitro models, employing rat duodenum, in which McN-A-343 stimulation of M1 receptors produces relaxation, and guinea pig paced atrium, where bethanechol evokes a negative inotropic effect by activating M2 receptors. Results showed that the guanyl analogue of ATR displayed affinities similar to those of the parent compound. A different behaviour was observed for the guanyl derivative of PZ. In fact, while affinity for M1 receptors was virtually unaffected, a significant decrease in KB value (15 folds) was found at M2 receptors. Such uneven effect on the recognition properties of compound is mirrored by a remarkable selectivity for the M1 receptor

compound	duodenum KB, nM	atrium KB, nM	selectivity ratio
ATR	1.2	1.8	1.5
guanyl-ATR	3.0	2.8	0.9
PZ	8.1	154	19
guanyl-PZ	7.8	2250	288

64 Direct measurement of muscarinic agonists and antagonists in the mouse central nervous system

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Cholinomimetics produce a range of side effects including hypothermia and tremor. Although these responses can be used to estimate the 'in vivo' activity of muscarinic agonists in the central nervous system (CNS), the approach is limited by compensatory feedback mechanisms and the difficulty of equating degree of receptor occupancy to effect. We have developed a novel 'ex vivo' assay to measure the potency and penetration of muscarinic agents into the CNS.

'Ex-vivo' binding was determined in brains of male PKTO mice (25g) that had been previously administered test compound. Whole brains were homogenised 30 mins later in ice cold 20mM HEPES buffer pH 7.4 containing 1mM MgCl₂. 100μl aliquots of homogenate were subsequently assayed with 0.6nM [3H] oxotremorine-M in a final volume of 1ml. Following a 40 min incubation at 30°C samples were filtered over Whatman GF/C filters. Specific binding was defined with 1μM atropine. Penetration of compound was calculated by comparison with a calibration curve added to homogenates of saline treated animals. Results are shown below and were compared with the ability of these compounds to induce (ED₅₀)/antagonise (ID₅₀) centrally mediated hypothermia.

Compound	Hypothermia ED$_{50}$/ED$_{50}$* (mg/kg)	Ex Vivo Binding ED$_{50}$ (mg/kg)	% Penetration into CNS
Pilocarpine	8.0 ± 3.3	21 (15;30)	0.31 ± 0.096
RS86	1.0 ± 0.5	11 (7.7;16)	1.5 ± 0.18
Scopolamine*	2.0 ± 0.9	2.6 (2.1;3.1)	0.21 ± 0.04
N Methyl Scopolamine*	10 ± 2.1	26 (20;32)	0.0059 ± 0.0013

Results in brackets refer to range (n=3). All other values are geometric mean ± SEM.

These results confirm that 'ex vivo' binding represents a reliable biochemical method of determining the potency and penetration of muscarinic agents into the central nervous system.

65 Identification of a small intracellular region of the rat m3 receptor responsible for selective coupling to PI turnover

J. Wess, M. R. Brann and T. I. Bonner. National Institutes of Health, Bethesda MD 20892, USA.

Molecular cloning studies have demonstrated the existence of five different muscarinic receptor proteins (m1 - m5). While m1, m3 and m5 are coupled to stimulation of phosphatidyl inositol (PI) metabolism, m2 and m4 are more efficiently linked to inhibition of adenylate cyclase. An analysis of the sequences of m1 - m5 reveals that a 16-17 amino acid segment at the amino terminal end of the third cytoplasmic loop (i3) is highly conserved among m1, m3, and m5 but different from the sequence which is well conserved in m2 and m4 . In order to investigate the role of this region in coupling specificity, we constructed cDNAs encoding chimaeric m2/m3 receptors, in which the whole i3 loop and the small i3 fragment, respectively, have been exchanged between the human m2 (Hm2) and the rat m3 (Rm3) receptor. Transient expression of these cDNAs in COS-7 cells showed that wild type Rm3 and the Hm2 receptor hybrids containing either the whole i3 loop or the short i3 fragment of Rm3 strongly couple to PI turnover, carbachol leading to a 2 to 3-fold increase in IP$_1$ production in the micromolar dose range. On the other hand, wild type Hm2 and the Rm3 receptor hybrids with the whole i3 loop or the short i3 segment of Hm2 induced less than 0.3-fold increases in IP$_1$ levels. These data strongly suggest that the short segment comprising 17 amino acids of the amino terminal end of i3 is sufficient to determine the coupling specificity of muscarinic receptors.

66 Location of sulfydryl groups of the muscarinic acetylcholine receptors involved in intramolecular disul'ide bonds by peptide mapping studies

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In purified muscarinic acetylcholine receptors (mAChRs) the ligand binding activity and the proportion of the sites with high affinity for agonists were affected by -SH reagents (Bernstein et al., J. Neurochem. 50, 1687-1694, 1988)

Eight cysteine residues are common to all five rat muscarinic receptor subtypes but the number and location of the S-S bridges is unknown. Recent studies show that two conserved aspartate residues in the mAChRs can be alkylated with ^{3}H-PrBCM One of these residues is immediately C terminal to a conserved cysteine which is probably involved in a S-S bond. Extended proteolysis of the denatured, reduced and carboxymethylated mAChR alkylated with ^{3}H-PrBCM, using lys-C, yielded two major glycosylated labelled products (28 and 54 KDa). The polypeptide molecular weights estimated after enzymatic deglycosylation were 14 and 22 KDa. In contrast, cleavage with lys-C

of non-reduced receptor followed by deglycosylation showed products with molecular weights of 22 KDa and 34 KDa. However, after further reduction and carboxymethylation, the original pattern of peptides was regenerated. This suggests that the 14 KDa peptide extends a disulfide bond which links it to another proteolytic fragment. In the present study the sulfhydryl groups of mAChRs involved in intramolecular disulfide bridges were labelled with ^{3}H-N-ethyl-maleimide (^{3}H-NEM).

Cleavage of the ^{3}H-NEM labelled mAChRs using lys-C yielded two distinct populations of labelled products. One population seems to correspond to the labelled glycosylated peptide residues obtained after proteolysis of the ^{3}H PrBCM-mAChRs (28-34 KDa peptides). This high molecular weight fraction probably contains a cysteine residue involved in a disulfide bond. Preliminary studies using a small amount of this population submitted to a second cleavage with CNBr, show the same peptides molecular weights as given by the ^{3}H-PrBCM-mAChRs in the same condition. The fractionation of the low M.W. peptides obtained from the COOH terminal of the mAChR should be a powerful tool to study the location of the other probable S-S-bridge(s) in the structure of the mACh receptors

67 Distribution of m1-m5 muscarinic receptor mRNAs in rat brain[1]

D. M. Weiner and M. R. Brann. Laboratory of Molecular Biology, NINDS. NIH, Bld. 36, Rm 3D-02, Bethesda MD 20892, USA and Research Scholars Program, HHMI, USA.

For each of the five muscarinic receptor mRNAs we prepared three 48 base oligodeoxynucleotide probes which are subtype specific. The specificity of these probes was verified based on each of the three probes independently labeling the same pattern of mRNA using in situ hybridization to consecutive sections through rat brain, and by northern blot analysis. The distribution of the mRNAs was determined with a mixture of the three probes, to increase specific activity and thus decrease autoradiographic time. Strong signals were observed for all of the receptor mRNAs within four weeks of autoradiographic exposure. Each of the mRNAs have a distinct distribution. For example, m1 mRNA is most abundant within the cerebral cortex and hippocampus (fields CA1-4 and dentate gyrus); m2 mRNA in the basal forebrain, septum and hindbrain; m3 mRNA in the cerebral cortex and hippocampus (fields CA1-4); m4 mRNA in the caudate putamen, nucleus accumbens and olfactory tubercle; m5 mRNA in the hippocampus (fields CA1-2), substantia nigra, pars compacta and globus pallidus. Data at a cellular level of resolution will be presented.

68 Regional expression of muscarinic receptor (MChR) subtypes in rat brain: an *in-situ* hybridization/receptor autoradiography study

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We have combined in situ hybridization histochemistry with synthetic oligonucleotide probes for the M1, M2, M3, M4 MChR (Bonner et al.(1987) Science 237:527-532) and receptor autoradiography using [^{3}H]-N methylscopolamine (NMS) as radioligand and several MChR antagonists (pirenzepine, hexahydrosiladifenidol, methoctramine, and AFDX 116) with differential receptor subtype selectivity to examine the microscopic distribution of these receptors in the rat brain. Forebrain areas such as the neocortex, hippocampus, and caudate-putamen exhibited high levels of hybridization for the M1 and M3 probes, whereas the levels were lower with the M4 probe.

M2 transcripts were at very low copy number and only seen in medial septum and pons. M3 transcripts were enriched in thalamic and midbrain areas, regions where M1, M2, and M4 mRNAs were not detected. In our preliminary binding studies we have not always found a good agreement between the presence of mRNA and the differential displacement of NMS binding by the antagonists. Thus, in the hippocampus, a majority of M1 receptor binding was found in spite of the presence of M3 and M4 mRNAs. Finally, sites in the midbrain (superior colliculus) were found to belong to the M3 class, in good agreement with the hybridization studies. Thus, the combination of these two autoradiographic approaches allows for the detailed delineation of the distribution of the cell bodies and binding areas where the different MChR subtypes are preferentially expressed.

69 Agonist pharmacology of cloned muscarinic receptors[1]

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Five muscarinic receptors (m1-m5) have been cloned, sequenced and expressed in mammalian cell lines. We have examined the pharmacology of m1-m4 receptors expressed in A9 L cells by measuring cAMP levels. When stimulated by agonists, m1 and m3 cause a rise in cAMP levels by coupling with a pertussis toxin-insensitive G-protein, while m2 and m4 cause a decrease in cAMP levels by coupling to a pertussis toxin-sensitive G-protein. Acetylcholine (ED_{50}s μM: m1, 4; m2, 1.5; m3, 5; m4, 0.3), carbachol, oxotremorine-M and muscarine were pure agonists at all of the tested receptors, and displayed ~ 10 fold lower ED_{50}s for the m2 and m4 than the m1 and m3. Arecoline, McN-A-343, pilocarpine (ED_{50}s μM / % maximum for carbachol "%M" : m1, 10 / 25%; m2, 0.5 / 80%; m3, 18 / 20%; m4, 0.5 / 65%) and RS 86 were partial agonists with lower ED_{50}s and greater efficacy at the m2 and m4 than at the m1 and m3. Maximal m1 responses to carbachol were more sensitive to receptor depletion by NPBC mustard than were m4 responses. These data are consistent with the selectivity between receptor subtypes of the above agonists being due, at least in part, to receptor spareness. BM-5 was a potent agonist at m4 receptors (ED_{50} / % M: 1 / 75%) but had little effect at m1, m2 and m3 receptors (efficacy < 10% M).

[1] This work was supported in part by Receptor Genetics, Inc.

70 Drug selectivity and second messenger systems in the cloned M_1 muscarinic receptor (R)

W. R. Roeske, L. Mei, J. Lai, S. Waite, T Smith and H. I. Yamamura. University of Arizona, Tucson, USA.

We have studied the relationship between the M_1 muscarinic R density and the R-mediated hydrolysis of inositol lipids in cloned murine fibroblast B82 cells which have been transfected with the m_1 muscarinic R gene. Of the 7 clones examined, the M_1 muscarinic R densities in these cells characterized by $[^3H](-)$MQNB binding ranged from 12 fmol/10^6 cells in LK3-1 cells to 260 fmol/10^6 cells in the LK3-8 cells. Carbachol-/$[^3H](-)$MQNB competition curves for the LK3-1 cells (with low R density) had a Hill coefficient of one. The competition curves for carbachol in the clones with

higher R densities had Hill coefficients less than one and were best fitted by a computerized nonlinear least-squares regression program for the 2-site model. Concentration-response curves for carbachol-stimulated $[^3H]IP_1$ accumulation were also obtained. A significant correlation was observed between the density of the M_1 muscarinic R with high affinity for carbachol and the maximum $[^3H]IP_1$ accumulation in these cells. There is no significant difference among the EC_{50} values, the K_A values obtained by the Furchgott method and the K_H values of the carbachol/-$[^3H](-)$MQNB competition curves. By using the fluo-3 method to determine the $[Ca^{2+}]_i$ stimulation by carbachol, we found an EC_{50} that agrees with the K_H. Drug specificity for binding, $[Ca^{2+}]_i$ and hydrolysis of inositol lipids confirm an M_1 muscarinic R profile. We conclude: (1) The high affinity state for carbachol may be the functional state of the M_1 muscarinic R in the transfected B82 cells; (2) there was evidence for the existence of spare M_1 R in the clones with high R densities but not in those with low R densities.

71 Muscarinic receptor binding, phosphatidylinositol (PI) turnover and Ca^{2+} mobilization in cells transformed to selectively express rat m_1 muscarinic receptors

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Cloning, sequencing, and expression studies have shown that there are five subtypes of muscarinic receptors (m_1-m_5). Recently, rat m_1 muscarinic receptors were inserted into murine B82 cells resulting in a cell line (M1C2) with a high level of receptor expression. Using Scatchard analysis, $[^3H]$-QNB was found to bind with a Kd of 5.0 ± 0.5pM and a Bmax of 593 ± 19fmol/mg protein was determined. Muscarinic agonists and antagonists bound to the receptor with Ki values ranging from 0.08nM (atropine) to 7653nM (carbachol). Receptor occupation by muscarinic agonists resulted in the stimulation of PI turnover with full agonists such as carbachol producing maximal stimulation, and other agonists (e.g. arecoline) producing similar effects but of lower magnitude. Since production of inositol trisphosphate results in an increase in free Ca++ levels, intracellular Ca++ was measured by both fura-2 and ^{45}Ca++ efflux techniques. As with PI turnover, muscarinic agonists increased free Ca++ with antagonists reversing this effect. Thus, M1C2 cells possess the characteristics of m_1 muscarinic receptors and are proving a useful tool for studying receptor/effector mechanisms (M1C2 cells provided by Dr. J.C. Venter, NIH).

72 Physiological comparison of cloned muscarinic receptor subtypes expressed in CHO cells

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Transformed CHO cells which express comparable levels of the muscarinic receptor subtypes m3, m4 and m5 (Buckley *et al*, Mol. Pharmacol. 35 469, 1989), were examined for physiological responses. CHO cells were electrically silent apart from a small TTX sensitive sodium current, as determined with whole-cell patch clamp recordings. Activation of m3 and m5 receptors by ACh hyperpolarized the cell membrane, and increased

conductance. Current-voltage curves showed the ACh-induced conductance reversed at around -65mV and shifted to around -20mV on increasing extracellular potassium from 5 to 50mM. Outward ACh-induced currents recorded at -50mV were attenuated by addition of 1μM atropine, 5mM Ba or 5mM Co to the bathing medium. Addition of 5mM BAPTA intracellularly, prevented activation of the ACh-induced conductance increase. The results indicate that both m3 and m5 muscarinic receptors activated Ca-dependent potassium conductances in CHO cells. Inositol phosphate (IP) levels were elevated 6-7 fold over baseline by carbachol stimulation of m3 and m5 receptors. Intracellular application of 100μM IP3 and raised intracellular calcium mimicked the ACh-induced current responses in both m3 and m5 transformants, suggesting that IP3 mediates the increase in intracellular Ca which in turn activates the potassium conductances. Basal cAMP levels were also elevated by activation of m3 and m5, however the response to m3 was 3-5 fold greater than that for m5 receptors. Application of up to 5mM 8-bromo-cAMP had no electrically detectable effect, and thus, cAMP is not involved in production of the ACh-induced current responses. m4 did not elicit an electrically detectable response, however, the receptor was demonstrated to be functional, as forskolin-stimulated elevations in cAMP were reduced on application of carbachol to m4-transformed cells in concentrations ranging from 0 01 to 30 μM. Above these concentrations cAMP levels were elevated.

73 Regulation of muscarinic receptors expression by nerve growth factor in developing corticostriatal cells in culture

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Recent studies suggest that nerve growth factor (NGF) exerts a trophic effect on forebrain and striatal cholinergic neurons and stimulates centr.   "aptic cholinergic markers.Changes in the expression of m.   cholinergic receptors (mAChR) by NGF was studied in dev   cort.costriatal cells in culture. This culture pr.   contains a small population of choline acetyltransfera.   itive neurons and synthetize from the third day n v   (DIV) increasing amount of ACh. M/ChR density, determined b   measuring 3H-QNB binding to cort/.al cell homogenate, increases by approximately 5 times during 12-13 days of culture. NGF treatment of cortico-striatal cells (0.1 ug/ml) elevates intracellular content of ACh (measured by HPLC-ED) and the activity of ChAT by 60-100% at the 8th-10th DIV. Results indicate that NGF treatment of corticostriatal cells induces a dramatic increase of mAChR number (Emax) that is accompanied by a 50% decrease of the Kd. The increase of 3H-QNB binding is dose-dependent and occours between the 9th and the 11th DIV, being maximal (30% above controls) at the 10th DIV. At the 12th DIV both control and NGF-treated cells contain the same maximum levels of mAChR number (approximately 500 fmol/mg prot.). Our working hypothesis is that NGF, by stimulating the activity of cholinergic neurons, regulates the expression of mAChR during development of corticostriatal cells in cultures. Preliminary results indicate that this culture preparation expresses messenger RNAs encoding for M1, M2, M3 and M4 mAChR subtypes, measured by reverse transcriptase-polymerase chain reaction (RT-PCR). We are currently investigating the effect of NGF on the expression of mRNAs encoding for muscarinic receptor subtypes.

74 Carbachol stimulation causes inhibition of mitogenesis and cell elongation in CHO cells transfected with muscarinic receptor genes

Bruce R. Conklin, Mark R. Brann, Noel J. Buckley, Tom I. Bonner, Alice L Ma, Christian C Felder and Julius Axelrod National Institutes of Health,HHMI, Bethesda, MD, 20892, USA

A family of genes encoding five distinct muscarinic receptors (m1 m5) have been cloned and stably expressed in fibroblasts (Bonner et al, Science, 237, 527-532: Neuron, 1, 403-410). We have previously reported that carbachol causes the inhibition of mitogenesis in AS L cells transfected with m1 and m3 receptors while m2 and m4 stimulation has no effect on mitogenesis in these cells (Conklin et al PNAS, 85, 8698 8702) We have now examined similar responses in CHO cells which have been transfected with m2-m5 receptors (Buckley et al, Mol Pharm 35, 469-476). Mitogenesis, as measured by thymidine incorporation, was inhibited by carbachol stimulation of m3, m4, and m5, but not the m2 transfected cells (see below). CHO cells are known to undergo cell elongation when treated with cholera toxin or cAMP analogs. We observed a similar cell elongation with carbachol stimulation of m3 and m5 transfected CHO cells Stimulation of the m2 or m4 transfected cells had little or no effect on morphology The elongation effect of the m5 receptor could be quantified, yeilding an EC-50 for carbachol of 63 nM (Felder et al, FEBS lett , 245, 75-79) The m5 stimulated elongation does not appear to be due to a rise in cAMP since the EC-50 for carbachol stimulated cAMP is 150 fold greater (10uM) The effects of these receptors on the growth and morphology may reflect biochemical differences in the actions of these receptors which are being elucidated in further studies

	m2	m3	m4	m5
% mitogenesis inhibition (thymidine incorporation)	3±3	47±2	17±4	27±3
cell elongation	-	+++	+/-	+++

Note: Although the m2 transfected CHO cells had second messenger effects with carbachol stimulation, the results may not be directly comparable because the m2 transfected cells expressed approximately one tenth the number of receptors as the m3, m4, and m5 transfected cells

75 Muscarinic receptors that mediate the enhancement of the rat vas deferens neurogenic contractions by cholinergic agonists: a new subtype?

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Cholinergic agonists augment the neurogenic contractions of the rat vas deferens neurotransmission via activation of muscarinic receptors. At last five subtypes of muscarinic receptor that differ pharmacologically functionally, and molecularly have been described inthe rat. In order to characterize the effector systems involved in the muscarinic effect in the RVD neurotransmission, rats were treated either with pertussis toxin (PT) (10 g/kg/ i.v.) and studied three days later or with lithium chloride (LiCl) (7 mmol/kg/day, i.p. for 3 days) and studied 24 hours after the last injection. PT treatment failed to alter the enhancing effect of Carbachol (CCh) in the RVD neurotransmission while it decreased the potency of CCh to produce the inotropic negative effect in the isolated atria of the same animals. LiCl treatment did not affect CCh action 'n RVD neurotransmission although it decreased the efficacy of this agonist to produce contractions of the isolated ileum from the same animals. We also determined whether the muscarinic receptors of the RVD under study exhibit plasticity when exposed to chronic blockade. In aged rats (18 months) atropine treatment (100 mg/kg/day, i.p. for 14 days) enhanced the potency of CCh to augment the RVD neurotransmission while it did not modify CCh action in the isolated atria or ileum from the same animals. In conclusion CCh enhancing action of the RVD neurotransmission appears to involve neither a pertussis toxin sensitive G protein nor the phosphatidyl inositol phosphate system. In addition, in aged rats, the receptors mediating this effect appear to up-regulate under conditions of chronic blockade.

76 Chemical and biochemical studies of restricted rotation isomers of BM-5

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Senile Dementia of the Alzheimer's Type (SDAT) is a neurodegenerative disease which results in the progress-ive impairment of memory. Autopsies of brain tissue from SDAT patients have shown a marked decrease in choi-inergic neurons. Taken together these observations form the basis for the cholinergic hypothesis for memory loss. A series of chemical synthesis projects have been init-iated in our geriatric program to discover selective cholinergic agonists to ameliorate the symptoms of this degenerative disease. From the chemist's point of view, the search for a useful pharmacological agent begins with the synthesis of a selective agent. Several cholinergic agonists and partial agonists are known which can be used as models or leads for a chemical synthesis project. One of these, N-methyl-N-(1-methyl-4-pyrrolidino-2-butynyl)acetamide (BM-5) has been reported [1] to be a presynaptic antagonist (which should disinhibit the release of endogenous acetylcholine) and a postsynaptic partial agonist (which should mimic the effects of acet-ylcholine). Chemically BM-5 is a flexible molecule that can assume a number of different conformations. The present research describes the asymmetric synthesis of a series of derivatives of BM-5 in which one degree of freedom has been restricted systemically.

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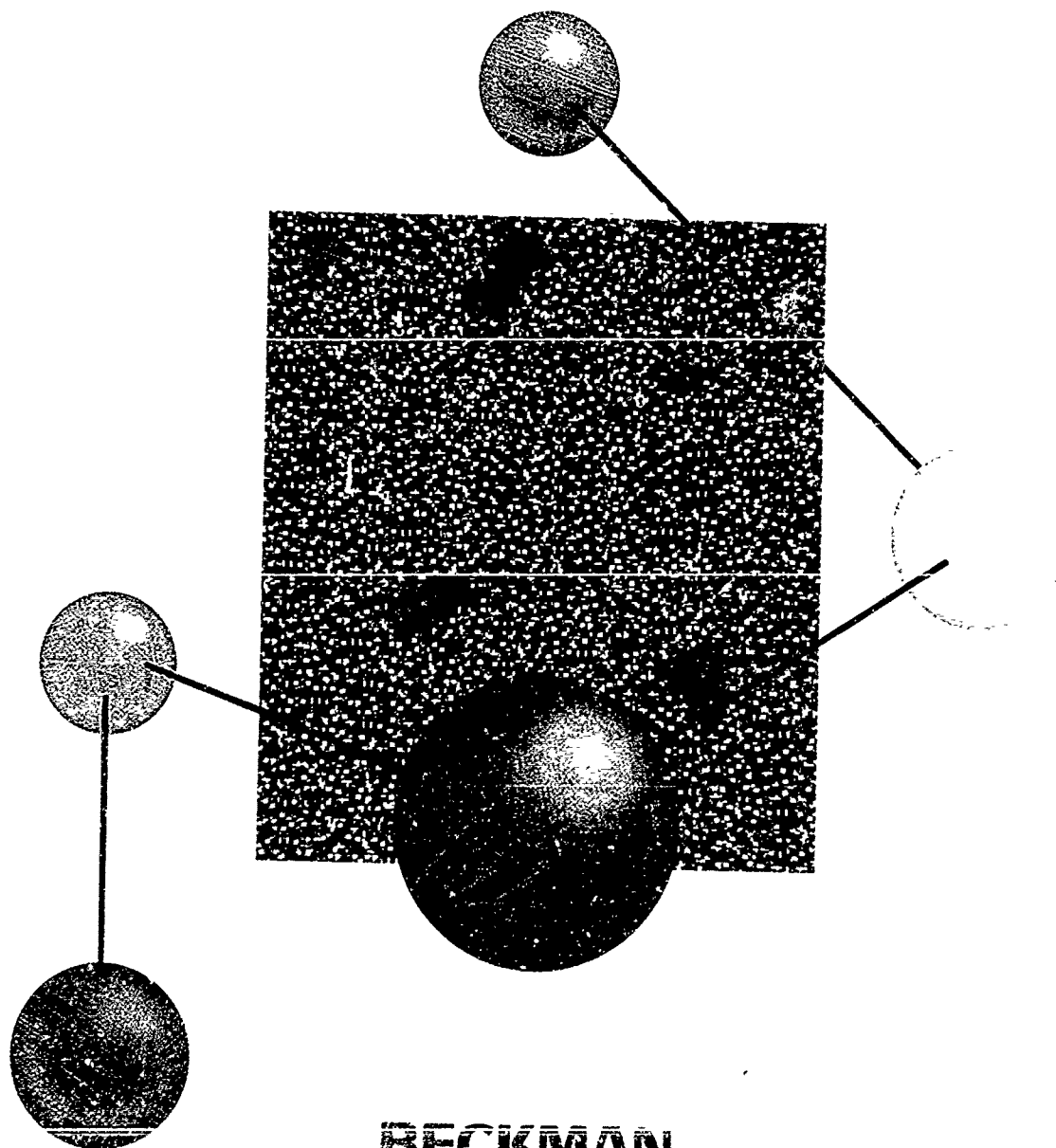
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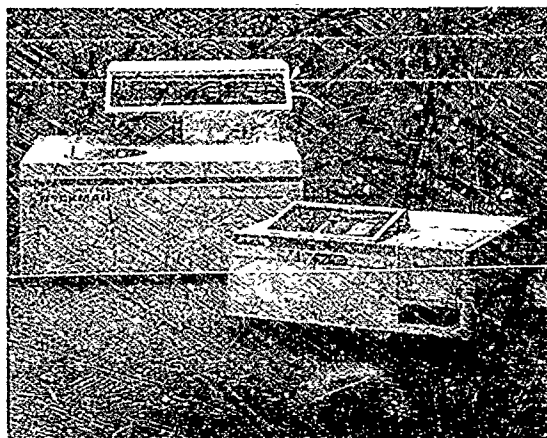
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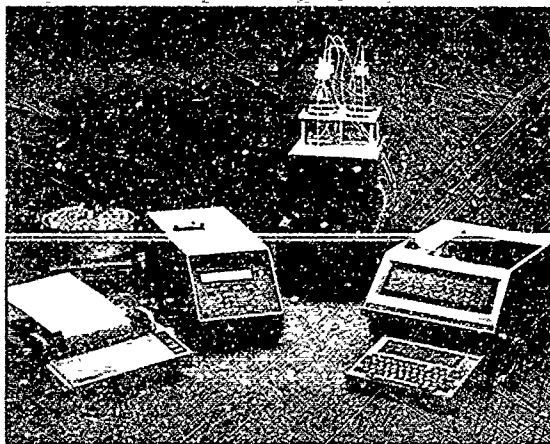

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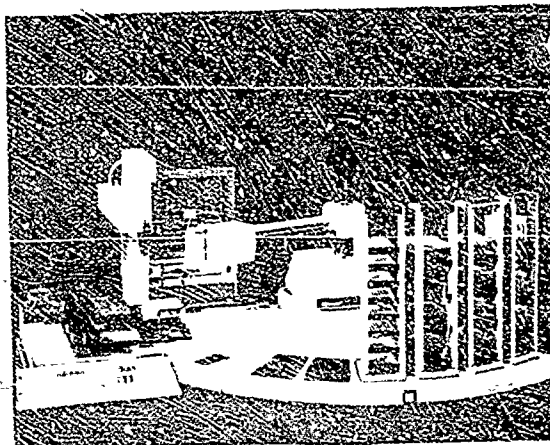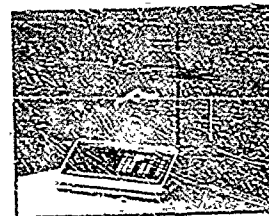
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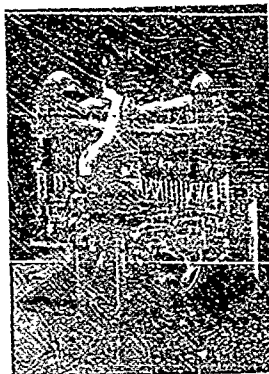
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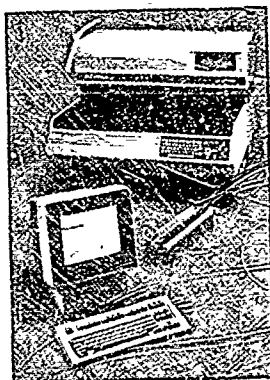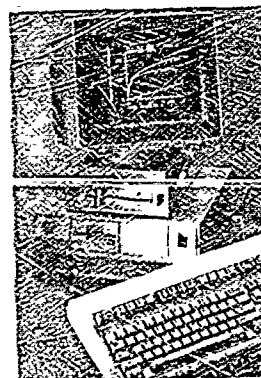
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Italy, Milan 2-69911
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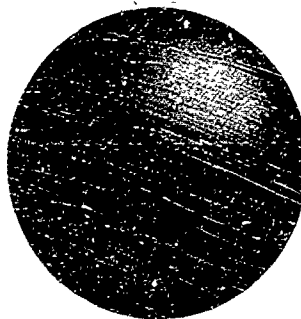
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